2009

Sialic Acid Conjugated Chitosan for the Attenuation of Amyloid-beta Toxicity

Dhruva Dilip Dhavale
Louisiana State University and Agricultural and Mechanical College, dhavale.dhruva@gmail.com

SIALIC ACID CONJUGATED CHITOSAN FOR THE ATTENUATION OF AMYLOID-BETA TOXICITY

A Thesis

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Master of Science in Chemical Engineering

in

The Department of Chemical Engineering

by
Dhruva D. Dhavale
B.E. University of Pune, Pune, India 2006
December 2009

To,

My Parents

Because of whom, I am what I am

ACKNOWLEDGEMENTS

I take this opportunity to express my immense sense of gratitude towards my advisor, Dr. James Henry. Thank you for supporting and encouraging me. He has been awesome in so many ways that is impossible to enumerate. Without his help, this work would not have been possible. I am fortunate to work under his guidance.

Thank you, Dr. Thibodeaux for believing in me. I would also like to thank my committee members, Dr. Benton and Dr. Monroe for their efforts in reviewing and evaluating my research.

I owe a great deal to my colleague and labmate, Mia Dvora. Thank you for introducing me to lab methods and protocols. Your patience and support is highly appreciated.

Special thanks to Ms. Thaya from the School of Veterinary Medicine, LSU and Mr. Andrews for being very helpful and accommodating for the use of their instruments.

I would like to acknowledge the funding agencies, Louisiana State University and the Chemical Engineering staff for allowing me to use the resources and helping me to complete my work smoothly and successfully.

I want to thank my dear friend Supriya for being there always when needed. Special thanks to Akanksha for her suggestions and help during my thesis writing. Thank you, Shivkumar for being a great roommate. Thanks to my friends for making Baton Rouge home.

I would like to dedicate this work to my parents. Thank you both for allowing me to pursue my goals, teaching me to always succeed, and never admit defeat. Baba, you will always be the standard by which I will measure myself. Aai, it is because of your unconditional love and support that I am here. To my brother Puru, my best friend, I will always cherish the memories of us growing up.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

Amyloid-beta (Aβ), a 39 to 43 amino acid long peptide, is the primary species identified in senile plaques associated with Alzheimer's disease (AD) and has been implicated in the neurotoxicity associated with AD. It is believed that Aβ toxicity is mediated through the interaction with neuronal membranes. A variety of evidence indicates that 1) Aβ may bind to the cell surface sialic acids, 2) the affinity of this interaction is higher if the gangliosides or sialic acids on the cell surface are clustered, 3) the removal of the surface sialic acids attenuate Aβ toxicity. Based on this data, we hypothesized that a biomimetic compound could be synthesized which would reproduce the clustered sialic acid structure of the cell surface, having antibody-like affinity towards Aβ, thus competing with the cell surface for Aβ binding. Our technique relies on attacking the theoretical "bottleneck" region in the Alzheimer's process, i.e. the interaction of Aβ with neurons. This area can be considered as a bottleneck as there are several mechanisms that can transform the Aβ peptide into its toxic form. Also, the exact toxic form of Aβ peptide that attacks neurons is not agreed upon. However, it is agreed that preventing neuronal interaction prevents toxicity making the Aβ-cell interaction the "bottleneck" region.

To explore this hypothesis further, we developed different sialic acid labeled compounds of different valency or number of sialic acids per molecule to attenuate Aβ toxicity. For this purpose, chitosan was used as a carrier molecule for sialic acids. EDC along with Sulfo-NHS was used as a cross-linker to couple the sialic acids with chitosan, with control over the degree of labeling. After verifying the presence of sialic acids on chitosan, the ability of this sialic acid-chitosan complex to attenuate the toxicity of aggregated Aβ was investigated in-vitro. Preliminary results indicate that the complex synthesized is biocompatible. Also, the results suggested that the compound has Aβ toxicity attenuating properties. Further studies will help

elucidate the role of cell-surface sialic acids in Aβ toxicity. Drugs available today are merely symptoms alleviating and thus, these results can have implications in the design of intelligent compounds that can bind pathogenic Aβ for the treatment of Alzheimer's disease.

1. OVERVIEW

The name "Alzheimer's Disease" was coined after a German Neurologist, Dr. Alois Alzheimer, who in 1906, described the autopsy findings of his 55 year old patient who died following a course of progressive dementia (Dahm 2006). This condition is most commonly observed in about 10% of people over 65 years of age and more than 50% for those over 85 years of age. Today, Alzheimer's Disease (AD) is the leading cause of dementia in the aging population and it is estimated that almost 25 to 30 million people currently suffer from this neurodegenerative disease (Goedert and Spillantini 2006; Minati, Edginton et al. 2009; Pimplikar 2009). AD has a slow progression and patients need special care and attention which creates a large burden on the health care system in terms of both services and cost.

Today, AD has no known causes and no known cures. Though medicines are available in the market, they are simply symptom relieving and do not hinder or stop the progression of the disease. The pathological characteristics of AD are the presence of neurofibrillary tangles and amyloid plaques in the brain of those affected by the disease (Grundke-Iqbal, Iqbal et al. 1986; Patel, Henry et al. 2006; Patel, Henry et al. 2007; K. Iqbal 2008; Pimplikar 2009). The main protein component of the plaques is a 39 to 43 amino acid peptide called beta-amyloid (Aβ). Though Aβ is found in healthy brains, the levels drastically increase in an AD brain. A number of studies have confirmed that Aβ plays an important role in the pathogenesis of AD (Hardy and Higgins 1992; Walsh, Hartley et al. 1999; Hardy and Selkoe 2002; Pimplikar 2009).

Aβ is generated from the amyloid precursor protein (APP) by the proteolytic cleavage of γ-secretase and β-secretase (Parihar and Hemnani 2004; Minati, Edginton et al. 2009). The Aβ peptide has amphipathic character, with a hydrophilic region (N-terminal) and a hydrophobic region (C-terminal), and can self-assemble to form aggregates with various morphologies such as

dimers, oligomers, filaments, protofibrils and fibrils (Lin, Chen et al. 2008). It is proposed that Aβ aggregation is a nucleation dependent process (Huang, Yang et al. 2000). Earlier, the insoluble fibrils observed on the neuronal surface were thought as the main species causing neurotoxicity. However, recent finding are contrasting and suggest that these insoluble fibrils might actually be the protective mechanism for the more toxic species, the Aβ peptide oligomers (Soto 1999). Currently, these oligomers, protofibrils and ADDLs are currently the most aggressively pursued target for both diagnosis and therapeutic treatment of AD.

A number of mechanisms and hypotheses have been postulated to explain the pathways by which Aβ exerts neurotoxicity. Aβ is known to generate reactive oxygen species (ROS) which leads to calcium homeostatis (Parihar and Hemnani 2004). Aβ is postulated cause synaptic dysfunction, neuroinflammation, microglial activation (M.Hoozemans, Chafekar et al. 2006), increase in membrane fluidity (Zubenko, Cohen et al. 1987), all of which contribute to cell death. Most importantly, Aβ is postulated to interact with the cell membrane through hydrogen bonding and electrostatic interactions. Investigations by Yanagisawa et al. showed the presence of monosialoganglioside GM1-bound Aβ (GM1-Aβ) in the brains of AD patients which is not detected in non-AD brains (Yanagisawa 2007). The GM1 ganglioside is a prominent lipid component of the cell membrane. Moreover, it is postulated that GM1-Aβ can act as a seed for Aβ polymerization leading to AD (Yanagisawa, Odaka et al. 1995; Yanagisawa and Ihara 1998). The Aβ peptide interacts via the surface sialic acids present on the gangliosides and this interaction and binding affinity is higher if the gangliosides or sialic acid molecules on the surface are clustered (Kakio, Nishimoto et al. 2001; Kakio, Yano et al. 2004). The Aβ initially interacts with the cell membrane gangliosides, undergoes a conformational change in structure then acts as a seed for free Aβ to accumulate.

The diagnosis of AD is not definitive and most of the drugs available today are ineffective in curing the disease. A number of different approaches such as secretase modulators, secretase inhibitors, Aβ aggregation inhibitors are under active development (Parihar and Hemnani 2004; Klafki, Staufenbiel et al. 2006; Barten and Albright 2008; Brody and Holtzman 2008). Immunotherapy is a promising approach but has proved complicated (Brody and Holtzman 2008). Most of these approaches suffer from the fact that most of the mechanisms, causes, chain of events and agents involved have not been identified and completely understood.

A new promising approach is to target the theoretical "bottleneck" in the Alzheimer's progression, the interaction of Aβ with neurons. This region is considered as the bottleneck because there are several theorized environmental conditions that lead to the formation of Aβ fibrils. Additionally, the exact form of Aβ (3-mer, 5-mer, 12-mer, protofibrils, fibril, etc.) that interacts with the neurons is still unknown. However, we know that Aβ peptide interaction with the neurons occurs through the gangliosides or sialic acids in membranes. Hence, it would be beneficial to design cell membrane mimicking materials that have antibody-like affinity towards Aβ. These membrane mimics could compete favorably with the cell surface for Aβ binding, thereby reducing the free Aβ that interacts with neurons thus protecting them from Aβ toxicity.

The thesis focuses on the synthesis of such a membrane mimicking compound that is multivalent in sialic acids and non-toxic. We postulate that this compound could be used to effectively attenuate the toxicity of aggregated Aβ in-vitro. Based on inputs from previous works, we use chitosan as a backbone due to its excellent biocompatible and non-toxic properties. To mimic the sialic acids present on gangliosides esp. GM1, sialic acid was conjugated to chitosan by the use of a zero-length crosslinker EDC and sialic acid-chitosan complexes of different degrees of labeling were synthesized. Thus, we believe that by effectively

mimicking the cell surface, we can trick Aβ into binding to our mimic, thereby preventing Aβ-cell interaction.

Therapies involving clearance of aggregated Aβ has shown promise in both animal and humans experiments. There is evidence that Aβ sequestering agents in serum may actually be effective at reducing Aβ levels in the cortex. Thus, it is hypothesized that the sialic acid-chitosan complex can effectively mimic the cell surface and can also protect cells from Aβ. This can provide crucial insights into the treatment of AD.

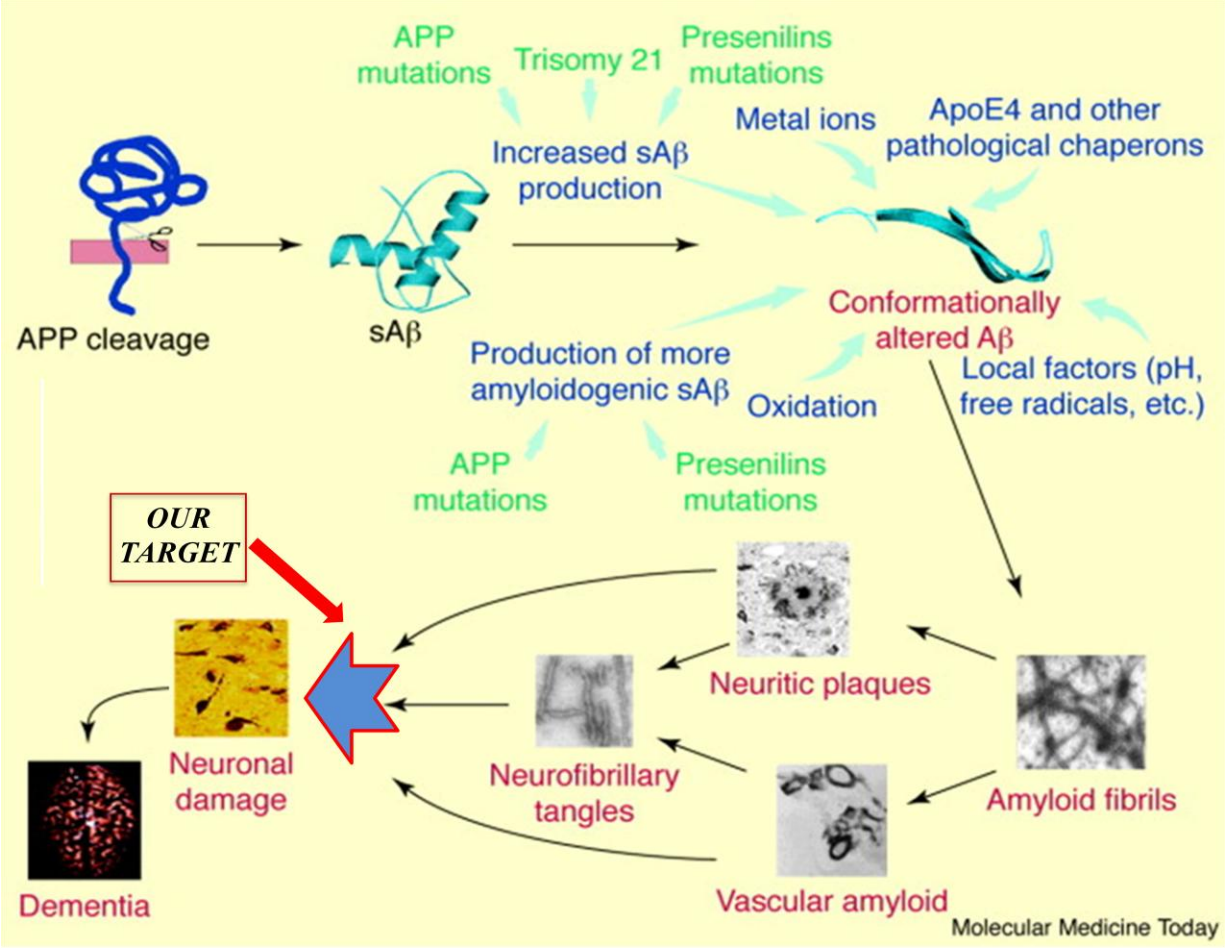

Figure 1: Theoretical hypothesis for the progression of Alzheimer's disease (Soto 1999), showing the bottleneck region that we plan to attack.

2. LITERATURE REVIEW

2.1. Introduction

On November 3, 1906, the audience at the annual conference of South-West German psychiatrists in Tübingen, were introduced to the first description of Alzheimer's Disease, when Dr. Alois Alzheimer talked about the psychiatric symptoms and the changed brain histology of his late patient, Auguste D (Dahm 2006). After several more cases were identified, similar to those first described by Dr. Alois Alzheimer, pathologists realized that they were dealing with a rather unique disease different from senile dementia (Berchtold and Cotman 1998). The official endorsement was made by Emil Kraepelin, a leading German psychiatrist who wrote: "That the involutional processes, known in man as old age, can also influence mental health seriously is most clearly demonstrated by the well known fact of senile dementia which in certain circumstances can lead to a progressive transformation and finally, to the destruction of the personality in the last decades of life" (Villemagne, Cappai et al. 2007). Kraepelin bestowed on this unique disease the eponymy of his colleague which we call Alzheimer's disease (AD).

A century later, AD is the most common cause of dementia and it is estimated that almost 25 to 30 million people currently suffer from this neurodegenerative disease (Goedert and Spillantini 2006; Minati, Edginton et al. 2009). In the latest statistics presented by the Alzheimer's Association, AD is the 6th leading cause of death in the US, with an estimated 5.3 million Americans of all ages suffering from AD, with projections that this number would triple by 2050 unless medical breakthroughs identify ways to treat and cure this disease. The annual cost for the direct and indirect care of AD patients is estimated at a staggering $148 billion (Alzheimer's Association 2009). Alzheimer's disease has a long and stressful clinical course in which the patients need special attention, ranging from home care to special nursing homes. Apart from its impact on the patients, this disease puts a significant physical, emotional and

financial burden to the families and relatives of the patients as well. Due to advances in science and medicine, the general life expectancy is increasing; making AD a problem of epic proportions that has to be addressed.

Despite over a century of research, AD still remains a complex disease which is not fully understood. There is no known cause and no known cure. One of the challenges now is to identify the cascade of events that lead to Alzheimer's disease. The progression of the disease is slow and the average period of survival is eight years, whereas some can survive for twenty years (Rauk 2008). The course of the disease depends on the health issues and the age at which diagnosis was done for the individual. The progression of the disease can be subcategorized into three stages (RD Terry 1994): In the first stage, some change in personality with decline in short term memory and beginning of faulty judgment is observed. The patient becomes less productive and spontaneous in everyday activities. The next stage results in more memory loss, impairment of language, attention and visuo-spatial and executive functions. Ability of a patient to perform day-to-day activities (eating, washing, grooming) start declining and the patient becomes more dependent on others. Short term memory becomes drastically impaired and only long established memories persist. Sleeping disorders, aggression, verbal outbursts and other troublesome behavior sets in. In the third stage, only fragments of memory remain. All cognitive functions are lost and the patient becomes mute, incontinent and eventually unresponsive to communication. The patient is at the mercy of the caregivers at this stage. Loss of immunity is the typical outcome making patients susceptible to infections which leads to death (Honig LS 2001).

Aging is the main risk factor of the disease (Harvey, Skelton-Robinson et al. 2003). Mutations in the amyloid precursor protein (APP) gene on chromosome 21, the presenilin 1 (PS1) gene on chromosome 14 and the presenilin 2 (PS2) gene on chromosome 1 have been

implicated in AD (Mayeux 2003; Luchsinger and Mayeux 2004; Minati, Edginton et al. 2009). Presence of apolipoprotein E (ApoE) e4 allele was shown to increase the risk of getting AD in conjunction with lowering the age of onset of the disease (Mayeux, Saunders et al. 1998; Rauk 2008). Other risk factors include decreased reserve capacity of brain (Blennow, de Leon et al. 2006), poor linguistic ability in early life (Snowdon, Kemper et al. 1996; Mayeux 2003), low mental and leisure activity (Lindsay, Laurin et al. 2002; Mayeux 2003), traumatic head injury (Mayeux 2003; Jellinger 2004), cardiovascular diseases like hyperlipidemia, hypertension, diabetes, obesity etc. (Mayeux 2003; Blennow, de Leon et al. 2006). [See (Mayeux 2003) for excellent review]

Alzheimer's disease affects the brain and is characterized by massive death of neurons and loss of synaptic connections throughout the brain (Alphonse Probst 1991). It starts in the hippocampus (an area of brain cortex responsible for new memories) (Rauk 2008), then spreads to the association areas of cerebral complex (responsible for language and reasoning) and finally to the neocortex (responsible for the sensory and motor area functionalities). This progression results in tissue loss throughout the brain thereby causing the brain to shrink in size and also the enlargement of the ventricles (fluid filled spaces within the brain). Proteinaceous deposits are observed in both the intracellular and extracellular compartments of the brain. Researchers have shown that the intracellular deposits are composed of neurofibrillary tangles (NFT) which are primarily formed due to the hyperphosphorylation of the tau protein (Grundke-Iqbal, Iqbal et al. 1986; Iqbal, del C. Alonso et al. 2005; Pimplikar 2009). NFT's are intraneuronal bundles of paired helical filaments formed by the microtubules, but they are not specific to AD and are found in various other neurodegenerative conditions such as Frontotemporal dementia, Hallervorden-Spatz disease etc (Villemagne, Cappai et al. 2007). Amyloid plaques are

extracellular aggregates of the Aβ peptide and many researchers have found a direct correlation between the presence of these plaques and the severity of AD (Hardy and Higgins 1992; Hardy and Selkoe 2002; Minati, Edginton et al. 2009; Pimplikar 2009). Earlier, the large insoluble plaques were thought as the toxic species, but recent evidence suggests that it is the small oligomers that may be the toxic species. The real insight into the disease was after 1984, when Glenner and Wong identified the amino acid sequence of Aβ peptides (Glenner and Wong 1984; Masters, Simms et al. 1985).

In the treatment of AD, many researchers are targeting the production and the aggregation process of Aβ. Since its conception, a number of theories and hypotheses have been put forward. The two major hypotheses that have been postulated to explain the molecular mechanisms of AD are the cholinergic hypothesis and the amyloid cascade hypothesis (Parihar and Hemnani 2004; Roland Jakob-Roetne 2009). The amyloid cascade hypothesis is relevant to the work done in this thesis is discussed in the later sections. Also, there is much evidence that points to the central role of Aβ in AD that supports the amyloid cascade hypothesis. As of now, none of the hypotheses are perfect and can satisfactorily explain Alzheimer's disease but, they provide a conceptual framework and a valuable roadmap for all researchers. With more and more advances in science, the missing links and pieces are being identified. This will be a valuable tool which will aid researchers in accurate diagnosis and in designing therapeutics for the treatment and cure of AD. Finally, progress in defeating this disease is hampered by the fact that AD is a very complex disease whose exact mechanisms and pathways still remain a mystery. The following sections will be devoted to understand some of the aspects of AD with an aim to design better therapeutic for its treatment.

2.2. Causes of AD

Researchers have identified the two main hallmarks of AD: the deposition of neurofibrillary tangles (NFT) composed of tau protein and the aggregation deposition of senile plaques comprised majorly of the amyloid-β peptide.

2.2.1. Tau Protein

Microtubules play an important role in maintaining the structural and physiological integrity of neurons. The biological activity of tau in promoting assembly and stability of the microtubules is regulated by its degree of phosphorylation (Iqbal, del C. Alonso et al. 2005). Evidence have shown that abnormal hyperphosphorylation of tau protein disrupts the microtubule structure resulting in the aggregation of tau into bundles of paired helical filaments (PHF), twisted ribbons and/or straight filaments collectively called neurofibrillary tangles (Iqbal, del C. Alonso et al. 2005; Li, Chohan et al. 2007; Barten and Albright 2008). Glycogen synthase kinase-3 (GSK-3) and cyclin dependent protein kinase-5 (cdk5) are the major protein kinases that have been implicated in the abnormal hyperphosphorylation of tau (Ishiguro, Omori et al. 1991; Iqbal, del C. Alonso et al. 2005; K. Iqbal 2008).This abnormal deposition of tau is observed in several other human neurodegenerative disorders and not just in AD. The NFTs are known to be toxic to the neurons which slowly and progressively lead to their death. Studies from different groups suggests that hyperphosphorylation of tau can be considered as one of the primary cause of AD, but not the fundamental one (Grundke-Iqbal, Iqbal et al. 1986; Iqbal, del C. Alonso et al. 2005; Stokin, Lillo et al. 2005). Thus, inhibition of abnormal hyperphorphorylation of tau protein is one of the most promising approaches for the development of therapeutic drugs. Drugs which inhibit GSK-3 and cdk5 have been developed by the industry and many of them are at different phases of clinical trials. Other strategies include

inhibiting the misfolding of tau and to directly stabilize the microtubules. As hyperphosphorylated tau is toxic by sequestering, normal mitogen activated proteins (MAPs), small molecules that can compete with this sequestering are being developed that can effectively attenuate toxicity of tau (K. Iqbal 2008).

Following diseases can be characterized by abnormal hyperphosphorylation of tau (Iqbal, del C. Alonso et al. 2005).

- Alzheimer's disease, including tangle-only form of the disease
- Down syndrome, adult cases
- Guam parkinsonism dementia complex
- Dementia pugilistica
- Pick disease
- Dementia with argyrophiliv grains
- Fronto-temporal dementia
- Cortico-basal degeneration
- Pallido-ponto-nigral degeneration
- Progressive supranuclear
- Gerstmann- Strä ussler- Scheinker disease with tangles

2.2.2. Amyloid-β Peptide

A common pathogenic mechanism in many different neurodegenerative disorders including AD is the aggregation and deposition of misfolded proteins mostly in the brain. As summarized in Table 1, nearly every major neurodegenerative disease is characterized by the

insidious accumulation of insoluble filamentous aggregates of normally soluble proteins in the central nervous system (CNS).

Table 1: Common Neurodegenerative Diseases Caused by Deposition of Aggregated Proteins (Skovronsky, Lee et al. 2006)

Disease	Microscopic lesion	Location	Aggregated protein
Alzheimer's Disease	Amyloid Plaque	Extracellular	Amyloid-β (Aβ)
	Neurofibrillary Tangle	Intracytoplasmic (neurons)	Tau
	Lewy bodies (seen in Lewy body variant)	Intracytoplasmic (neurons)	α-synuclein
Amyotrophic lateral sclerosis	Hyaline Inclusions	Intracytoplasmic (neurons)	Superoxide dismutase-1 (SOD-1)
Cortical basel degeneration/ progressive supranuclear palsy	Tau positive inclusions	Intracytoplasmic (neurons, oligodendroglia and astrocyes)	Tau
Dementia with Lewy bodies	Lewy bodies	Intracytoplasmic (neurons)	α-synuclein
Huntington Disease	Neuronal Inclusions	Intranuclear (neurons)	Huntington (With Polyglutamine repeat expansion)
Multiple system atrophy	Glial cytoplasmic inclusions	Intracytoplasmic (oligodendroglia)	α-synuclein
Parkinson's Disease	Lewy Bodies	Intracytoplasmic (neutrons)	α-synuclein
Pick's Disease	Pick Bodies	Intracytoplasmic (neutrons)	Tau
Prion Diseases	Prion Plaques	Extracellular	Protease-resistant prion protein (PrP)

These diseases are usually grouped together as the filamentous aggregates show similar ultra structural and tinctorial (staining or coloring) properties of amyloid (i.e. ~10nm wide fibrils with crossed β-sheet structures which stain with congo red, thioflavin-S or other related dyes). Hence, they are collectively known as brain amyloidoses (Skovronsky, Lee et al. 2006).

Glenner and Wong first identified the major protein component of vascular amyloid, a low-molecular weight, an approximately 4kDa polypeptide, now referred to as β-amyloid protein (Glenner and Wong 1984; Villemagne, Cappai et al. 2007). This protein was also found to be a major component of amyloid plaques (Masters, Simms et al. 1985; Villemagne, Cappai et al. 2007) which led to the identification of its precursor, the amyloid precursor protein (APP). Soon after the discovery, the APP gene was cloned allowing the disease to be examined at molecular levels. Subsequently, mapping of mutations in APP gene, the association of AD with Down's syndrome (People with Down's syndrome have an extra copy of chromosome 21, which also contains APP), higher prevalence of AD with increased copy number of APP and the identification of mutations in presenilin 1 (PS1) all confirmed the central role of Aβ peptide and APP in Alzheimer's disease (Hardy and Higgins 1992; Hardy and Selkoe 2002; Pimplikar 2009).

2.2.2.1. Characteristics of Aβ Peptide

Aβ, a 39 to 43 amino acid long peptide, is cleaved from the C-terminal region of the membrane spanning glycoprotein, the amyloid precursor protein (APP). APP is found in tissues throughout the body but its primary function is still unknown (Rauk 2008; Minati, Edginton et al. 2009). A large part of the APP lies in the ectodomain and contains the N-terminus, whereas its C-terminus is located in the cytoplasmic domain. APP has the characteristics of a cell surface receptor and is located on chromosome 21 (Minati, Edginton et al. 2009). The Aβ sequence itself comprises part of the ectodomain of the APP and extends into, but not all the way through, the transmembrane domain (Wilquet and Strooper 2004). Aβ contains 28 amino acids from the extracellular part of APP and the rest 11 – 15 residues are located in the transmembrane domain (Kremer and Murphy 2003).

There are two pathways by which the APP can be metabolized in the cells and tissues: the non-amyloidogenic and amyloidogenic pathway (Nathalie and Jean-Noel 2008). In the non-amyloidogenic pathway, the APP is cleaved by α-secretase between residues 687 and 688, which releases a soluble extracellular sequence (α-sAPP) and a membrane attached C-terminal fragment (CTFα). The CTFα is further cleaved at a variable position (between the C-terminus

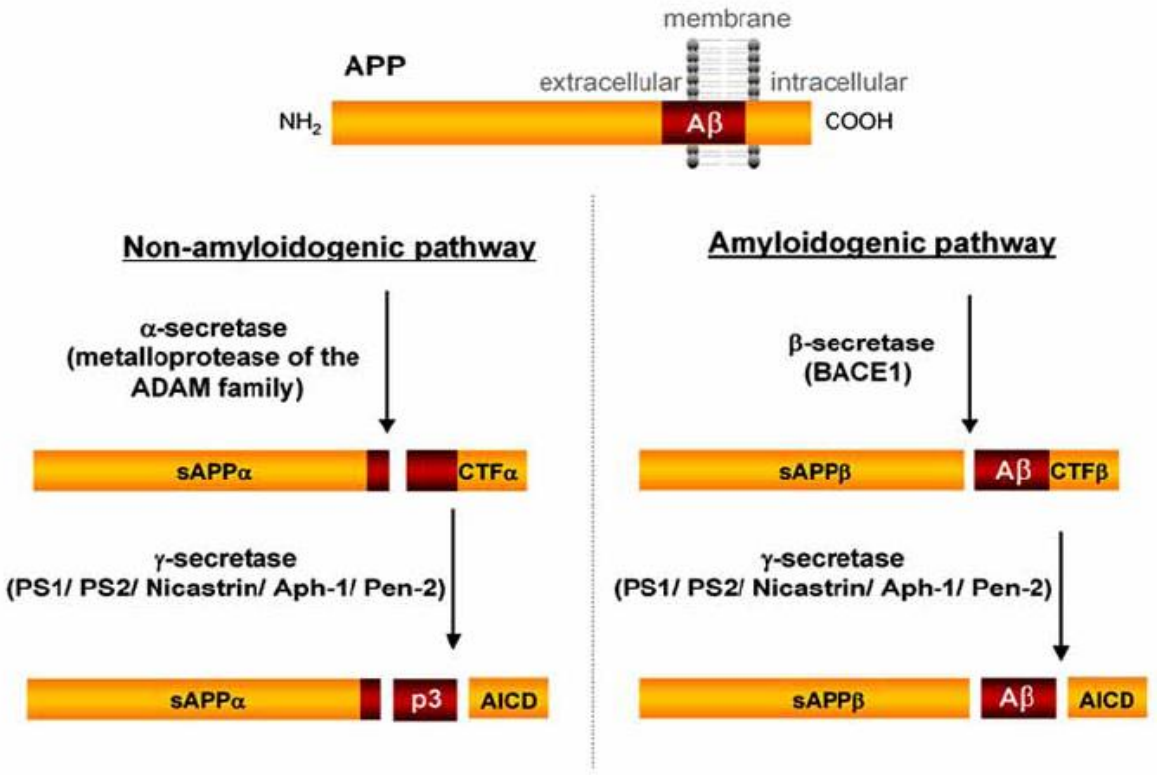

Figure 2: Metabolism of APP and the formation of Aβ peptide (Nathalie and Jean-Noel 2008). Reprinted with permission from Bentham Science Publishers Ltd.

and residue 712) by γ-secretase in the transmembrane region which releases the harmless, 3kDa, p3 fragment and the APP Intracellular C-terminal domain (ACID) (Nathalie and Jean-Noel 2008; Minati, Edginton et al. 2009). This cleavage of α-secretase takes place within the Aβ fragment, thereby preventing the release of the full-length Aβ polypeptide, hence referred to as the non-amyloidogenic pathway (Parihar and Hemnani 2004).

In the amyloidogenic pathway, the Aβ peptide is formed when the APP is cleaved by β-secretase in between residues 671 and 672 followed by cleavage in between the C-terminal and residue 712 by γ-secretase (Parihar and Hemnani 2004). Along with the soluble extracellular β-sAPP fragment, several isoforms of Aβ can be produced of which the 40 and 42 amino acid forms are the most common ones (Nathalie and Jean-Noel 2008; Minati, Edginton et al. 2009). Aβ(1-40) is the predominant species produced, whereas Aβ(1-42) accounts for only 10% of the total secreted Aβ. However, Aβ(1-42) is considerably more prone to aggregation and is regarded a more neurotoxic. The levels of Aβ(1-42) are believed to be elevated in AD. The Aβ(1-40) to Aβ(1-42) ratio can be influenced by several factors such as substrate concentration, PS1 and PS2 mutations and can have an effect on the formation of senile plaques.

Three different proteases appear to be responsible for the α-secretase activity: TACE (TNF-α converting enzyme), ADAM-9 and ADAM-10 (a disintegrin and metalloprotease domain protein). The protein responsible for β-cleavage has been identified as a movel transmembrane aspartyl protease BACE1 (β-site APP cleaving enzyme 1) and it is posited that levels of BACE1 increase in AD. As β-secretase is the Aβ producing enzyme, it is the ideal therapeutic target, but complete abolishment of BACE1, have shown deleterious effects in knockout mice (Cole and Vassar 2008). The enzyme γ-secretase is believed to be a complex of at least four proteins: Presenilin 1(PS1) or Presenilin 2 (PS2), Nicastrin, Pen-2 and Aph-1, but other protein components of this complex may also exist. Notch signaling is also affected by γ-secretase (Nathalie and Jean-Noel 2008). It is the γ-secretase dependent cleavage that is affected by most missense mutations that cause excess production of Aβ(1-42). However, all these enzymes have not been completely identified. Much is unknown about the different substrates that the attack. Research have shown that α-secretase and β-secretase compete for the APP

substrate as increase in one pathway has shown decrease in other pathway and vice-versa (Cole and Vassar 2008).

In the Aβ sequence, the first 16 residues (N-terminal region) are found to be largely hydrophilic whereas the remaining residues (C-terminal region) form the hydrophobic domain. Thus, amphipathic Aβ has propensity towards self-aggregation and accumulation, which is supposed to initiate a cascade that triggers complex pathological reactions eventually leading to AD. The Aβ peptide forms various structures such as dimers, 5-mers, oligomers, protofibrils and fibrils through the aggregation process. It is the central region of Aβ(12-23) that has been implicated as the self-recognition site for the formation of dimmers and higher oligomers (Rauk 2008).

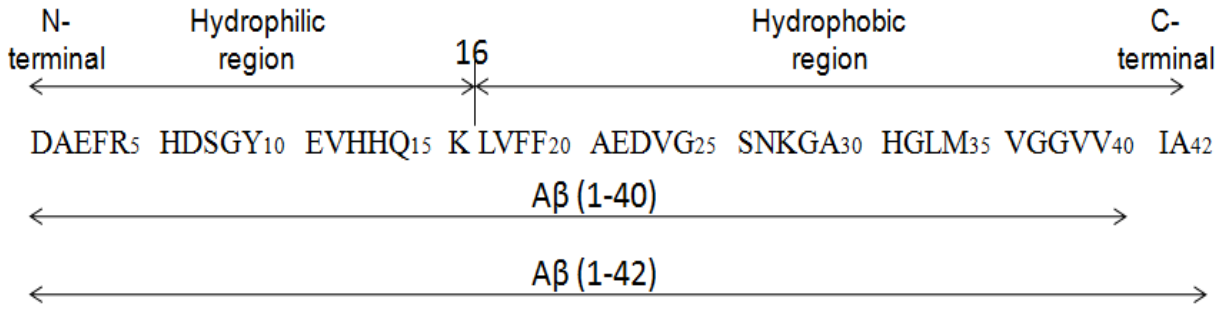

Figure 3: Sequences of Alzheimer's amyloid-β peptides (Glenner and Wong 1984; Bateman, McLaurin et al. 2007; Rauk 2008)

2.2.2.2. Evidence for Aβ Induced Neurotoxicity

It was observed that AD patients showed evidence of extensive oxidative stress (Rauk 2008) caused by reactive oxygen species (ROS) present in the brains (Praticò 2008). One of the sources of ROS is believed to be the Aβ peptide, which works in conjunction with metal ions and oxygen. It was also noted that oxidative stress also led to the over-expression and misprocessing of the APP gene, which further led to more production of Aβ. This results in a dangerous cycle that eventually leads to neuronal death and brain degeneration (Grundman and Delaney 2002).

In addition to oxidative stress, cell membrane permeability is severely compromised by Aβ peptide when it forms calcium permeable ion channels in the plasma cell membrane (Rauk 2008). These channels allow excess calcium influx and disrupt the normal calcium homeostasis. In-vitro studies by Lin et.al showed that Aβ(1-42) induced rapid neuritic degeneration at physiological nanomolar concentrations (Lin, Bhatia et al. 2001). Recent evidences have suggested that formation of ion-permeable pores maybe the condition before Aβ is released in the extracellular space.

Researchers have also shown that Aβ causes damage to the blood brain barrier (BBB) through the production of superoxide and the involvement of homocysteine (Rauk 2008). There is evidence suggesting that Aβ binds to an intracellular polypeptide called ERAB and its toxicity to neurons is directly related to the expression of ERAB (Du Yan, Fu et al. 1997). Aβ is involved in decreasing synaptic activity and causing progressive neuronal degradation. The fact that neuronal death is observed in the immediate vicinity of Aβ deposits further implicates Aβ in the pathogenesis of AD (Yankner and Lu 2009).

These factors have confirmed the central role of Aβ and APP in the etiology of the Alzheimer's disease. Since then much of the work in designing effective therapeutics for AD has focused on the Aβ peptide. A number of different hypotheses have been proposed, out of which, the amyloid cascade hypothesis presented by Hardy and Higgins (Hardy and Higgins 1992) have received the most attention (Pimplikar 2009). It is reviewed in the next section.

2.2.2.3. The Amyloid Cascade Hypothesis

In 1992, Hardy and Higgins presented the "amyloid cascade hypothesis" which explained the pathogenesis of sporadic AD. The hypothesis proposes that the increased production or decreased clearance of Aβ peptide is the fundamental cause of AD. They proposed that Aβ

causes the hyperphosphorylation of tau protein which starts the cascade of events leading to the formation of amyloid plaques and neurofibrillary tangles (Hardy and Higgins 1992).

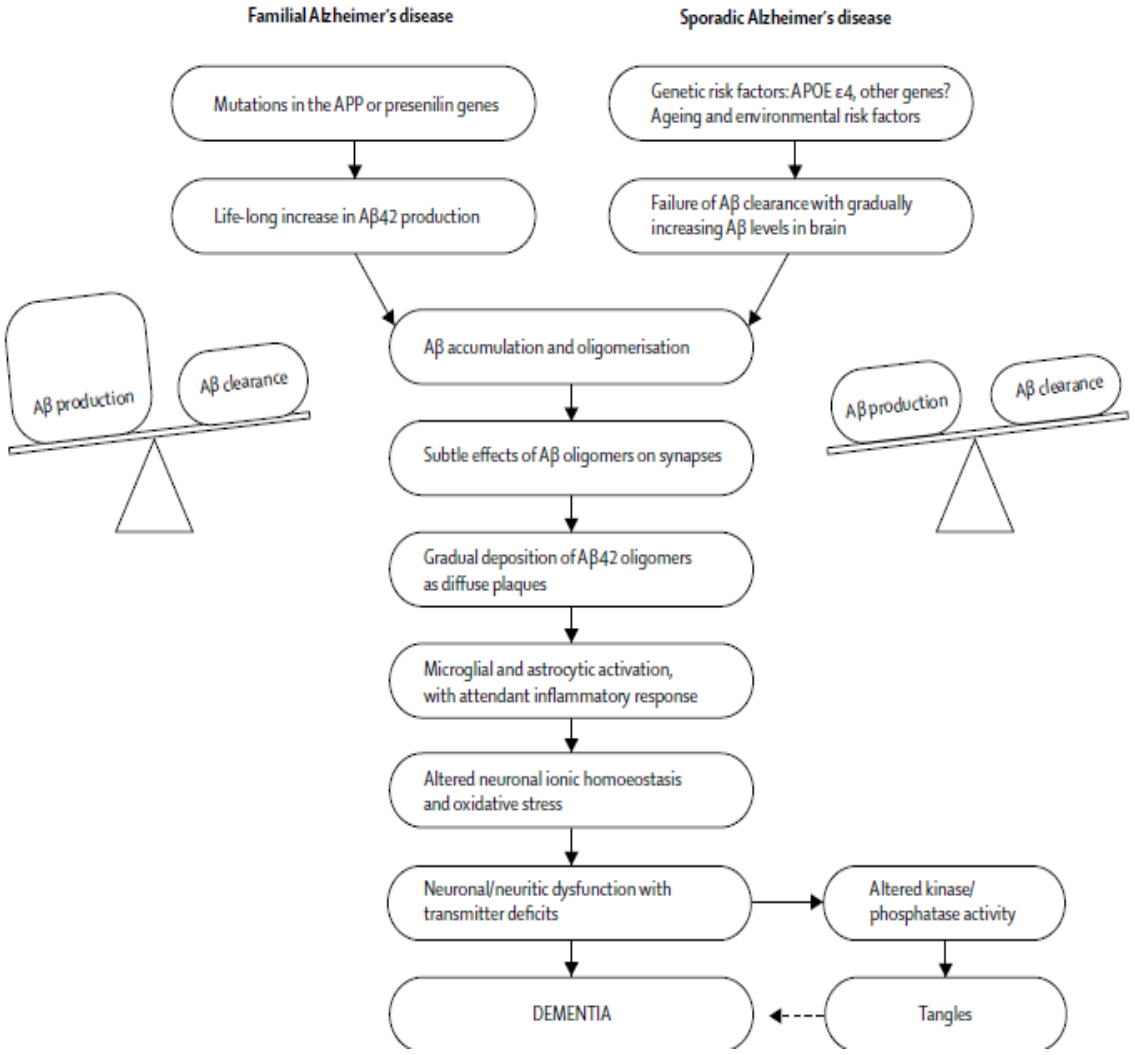

Figure 4: Mechanism of amyloid cascade hypothesis. The central event in the disease pathogenesis is an imbalance between Aβ production and clearance, with increased Aβ production in familial AD and decreased Aβ clearance in sporadic AD. This accumulation of Aβ leads to microglial activation and inflammatory response. The loss of homeostasis and oxidative stress leads to synaptic dysfunction, altered kinase activity affecting tau production. The NFTs and plaques lead to neuronal death and dementia. (Blennow, de Leon et al. 2006) Reprinted with permission from Elsevier Ltd.

Since then, the amyloid cascade hypothesis has undergone alterations as newer research finding are being presented. A decade after the hypothesis was originally presented, Hardy and Selkoe proposed an amended version which took into consideration the mutations in the APP,

PS1 or PS2 genes which increases Aβ production. This results in the accumulation of Aβ followed by the oligomerization and deposition of Aβ as plaques. These Aβ plaques cause increased synapse destruction, altered neuronal ionic homoeostasis and oxidative injury, which leads to hyperphosphorylation of tau protein and causing the deposition of NFT's and neuronal destruction (Hardy and Higgins 1992; Hardy and Selkoe 2002; Blennow, de Leon et al. 2006). Support for this theory includes the fact that AD brains demonstrate extensive Aβ deposition (Patel, Henry et al. 2006); mutation in the genes implicated in familial forms are all related to APP processing, which increases Aβ production (Scheuner, Eckman et al. 1996; Blennow, de Leon et al. 2006; Minati, Edginton et al. 2009; Pimplikar 2009); Down's syndrome patients (who have an extra APP gene) develop Aβ plaques early in life (Blennow, de Leon et al. 2006; M.Hoozemans, Chafekar et al. 2006; Tansley, Burgess et al. 2007) and several in-vitro studies have also demonstrated the neurotoxic nature of soluble Aβ oligomers to cells and applied them to animal models (Kayed, Head et al. 2003; Pimplikar 2009).

Crucially, the amyloid cascade hypothesis is not perfect and the exact mechanism of Aβ toxicity remains elusive as the specific neurotoxic species of Aβ and the nature of its effects on neuronal function have not been defined in-vivo. Earlier, it was thought that Aβ deposited as plaques were neurotoxic. However, recent finding demonstrate that soluble pre-fibrillar oligomers of Aβ are likely to be the toxic species that initiate neurodegeneration (M.Hoozemans, Chafekar et al. 2006).

2.2.2.4. Mechanisms of Aβ Neurotoxicity

A number of mechanisms have been suggested to explain the pathway by which Aβ induces neurotoxicity. Some of them are explained in brief below:

Studies have shown that Aβ binds to a metal substrate generating reactive oxygen species (ROS). Aβ also causes loss of calcium homeostasis that generates reactive nitrogen species (RNS). One plausible explanation is that the Aβ can enter the mitochondria, where it increases the production of ROS, significantly reducing the levels of antioxidants (e.g., vitamins E, C and gluthathione) thereby altering the balance in the brain (Rauk 2008). Depending on the substrate attacked, oxidative stress will manifest as protein, DNA, RNA oxidation or lipid peroxidation (Praticò 2008). These species are extremely reactive causing damages to DNA, RNA and oxidation of lipids and proteins. This creates an imbalance which leads to oxidative stress and induces inflammation in the neurons leading to their death (Multhaup, Ruppert et al. 1997; Parihar and Hemnani 2004; Rauk 2008). Figure 5 explains a possible hypothesis of how oxidative stress can lead to increased Aβ production and so contributing to neuronal death.

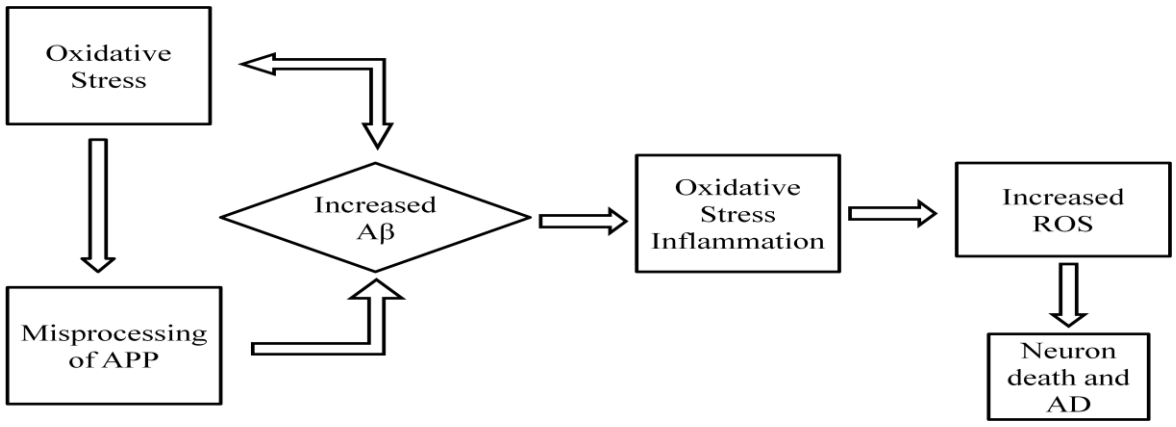

Figure 5: Possible mechanism of Aβ neurotoxicity due to oxidative stress

Another mechanism that has received considerable thought is that Aβ causes synaptic dysfunction. Neurons are connected to each other through junctions called as synapses and tiny electrical pulses are transmitted through these junctions as a means of communication between two neurons. It is believed that synaptic terminals are critically dependent on levels of cortical Aβ. After the onset of AD, levels of Aβ start rising which leads to synaptic dysfunction thereby inducing neurotoxicity (Selkoe 2002; M.Hoozemans, Chafekar et al. 2006).

Neuroinflammation and microglial activation is another possible mechanism of Aβ induced neurotoxicity. Aβ plaques have been found with clusters of microglia. Microglia are considered to be the brain resident macrophages responsible for the maintenance of homeostasis within the brain. They are activated when the brain detects signs of oxidative stress and neuronal damage. It is believed that the microglia cells are activated to clear the Aβ by phagocytosis. This process involves the release of ROS, pro-inflammatory cytokines, excitotoxins and proteases, all potentially neurotoxic substances (M.Hoozemans, Chafekar et al. 2006).

Interaction with tau protein is considered as another mechanism through which Aβ is thought to induce neurotoxicity. It is believed that Aβ(1-42) and ApoE4 activates various kinases that results in the hyperphosphorylation of the tau protein, which in turn form NFTs.

Aβ also adheres to endothelial cell walls forming damaged tissues or lesions. Over time, accumulation of Aβ deposits can lead to a condition known as cerebral amyloid angiopathy (CAA), which leads to internal bleeding in the brain. Aβ causes pore formation in the membranes that lead to loss of calcium homeostasis and an influx of Ca^{2+} into the neurons. This is believed to start a cascade of events which ultimately leads to neuronal death (Lin, Bhatia et al. 2001; Rauk 2008).

Other mechanisms proposed for Aβ neurotoxicity include increased membrane fluidity (Zubenko, Cohen et al. 1987), alteration of cytoskeleton and nucleus (Braak, Braak et al. 1994); redox active iron (Smith, Harris et al. 1997), binding of Aβ to ApoE and catalases. It is believed that several mechanisms might be active simultaneously and could be interrelated and dependent on each other. However, no consensus has been reached on a perfect mechanism for Aβ neurotoxicity, which makes the design of therapeutics for AD a difficult task.

2.2.2.5. Normal Roles of APP and Aβ

The exact roles of APP and Aβ in the normal functioning of cells are not fully understood. Aβ is secreted by neuronal cells as a part of normal metabolism. Ill effects such as lower weight, reduced locomotor activity and impaired neuronal functions in brains are observed in experiments on APP knockout mice (Zheng, Jiang et al. 1995). The APP intracellular domain (ACID) formed by the γ-cleavage of APP, is believed to regulate phosphodinositide-mediated calcium signaling, which plays an important role in cell differentiation (Findeis 2007). Studies have shown that Aβ(1-40) is produced as a cellular antioxidant (Teng and Tang 2005), Aβ(1-40) modulates potassium channels in neurons with Aβ(1-42) and it also counteracts the effects of secretase inhibitors (Plant, Boyle et al. 2003).

2.2.2.6. Aβ Neurotoxicity: Who Is the Real Culprit?

Aβ protein is derived from APP and is found to be present in the cerebrospinal fluid (CSF) and in brains of normal humans. Hence, the mere presence of Aβ cannot be the cause of dementia. However, ordered self-association of Aβ molecules seems to be the factor causing neuronal degradation (Walsh and Selkoe 2007). Self-association and aggregation of Aβ can lead to various forms of aggregates such as monomeric species, small dimers, oligomers, larger assemblies commonly referred to as Aβ-derived diffusible ligands (ADDL) or protofibrils and large insoluble fibrils.

Initially, the large insoluble fibrils, which deposited as plaques were considered neurotoxic because these fibrils were detectable and characterization of the assemblies that formed in-vitro were limited (Walsh and Selkoe 2007). However, the fact that amyloid fibrils are the AD causing species is frequently challenged as a weak correlation is found between the amount of plaques deposited and the severity of dementia in patients.

Recent evidences have suggested that soluble oligomers are likely to be the real culprits. Studies have shown that oligomeric Aβ, in the absence of monomers and fibrils resulted in toxicity in-vivo and oligomers-specific antibodies could block the toxicity in neurons. A strong correlation was found between the soluble Aβ levels, loss of synapses and severity of dementia, further implicating soluble Aβ as the toxic intermediate (Wang, Dickson et al. 1999; Walsh and Selkoe 2007). The term, 'soluble Aβ' describes the form of Aβ that can remain in solution even after high speed ultracentrifugation. Studies from synthetic Aβ peptides, APP over-expressed cell culture systems, APP transgenic mice and human CSF and postmortem brain have indicated that soluble non-fibrillar Aβ induces toxicity in cells (Walsh and Selkoe 2007).

Protofibrils (PFs) are a group of structures ranging from spheres (about 5nm in diameter) to curvilinear structures about 200nm in length. PFs are physically similar to amyloid fibrils but they have the ability to form both true amyloid fibrils or dissociate into low molecular weight species of Aβ. PFs and amyloid fibrils also have distinct biological activities (Walsh, Lomakin et al. 1997; Walsh, Hartley et al. 1999; Walsh and Selkoe 2007). Shortly after the discovery of protofibrils, Lambert et al. observed small (5-6nm) globular structures of synthetic Aβ(1-42) with the C-terminal region of Aβ forming a hydrophobic core, which they referred to as Amyloid-β derived diffusible ligands (ADDLs) (Lambert, Barlow et al. 1998). The ADDLs are the smallest assemblies of PFs, about 6nm and have been shown to cause neuronal death, block long-term potentiation (LTP), inhibit reduction of MTT in neural cells and avidly bind and decorate dendritic arbors of certain cultured neurons (Walsh and Selkoe 2007). A dodecamer, labeled Aβ*56 for its weight in kD, is proposed to induce memory loss independent of neuronal plaques before amyloid plaques started developing (Lesne, Koh et al. 2006). However, no consensus has been reached on the exact toxic species and it is thought that toxicity can be

induced by multiple assemblies rather than any particular form. However, the fact remains that in-vivo environment is quite different than the in-vitro environment and the hydrophobic nature of the Aβ peptide makes it ambiguous whether the pathway observed would also work in-vivo.

Now that the different assemblies of Aβ have been discussed, the pathways by which Aβ aggregates needs to be addressed. It is proposed that Aβ aggregation is a nucleation dependent polymerization process, which is significantly affected by the presence of small peptide aggregates or 'seeds' (Huang, Yang et al. 2000) and by the rate of elongation of the seeds (Walsh, Lomakin et al. 1997). Walsh et al. (Walsh, Lomakin et al. 1997) showed that Aβ oligomerises through a series of short lived intermediates that form PFs, which act as centers for the growth of mature insoluble fibers. Thus, the monomers can be in equilibrium with dimers to form fibril nuclei from which protofibrils emerge. The end to end or lateral association of PFs forms "self-templates", onto which the monomers/dimers bind and polymerize. Most of the other models proposed are variation of what is depicted in figure 6.

Huang et.al. (Huang, Yang et al. 2000) suggested two pathways for Aβ aggregation. In one pathway, an ordered β-sheet conformation is observed which leads to AD like symptoms and

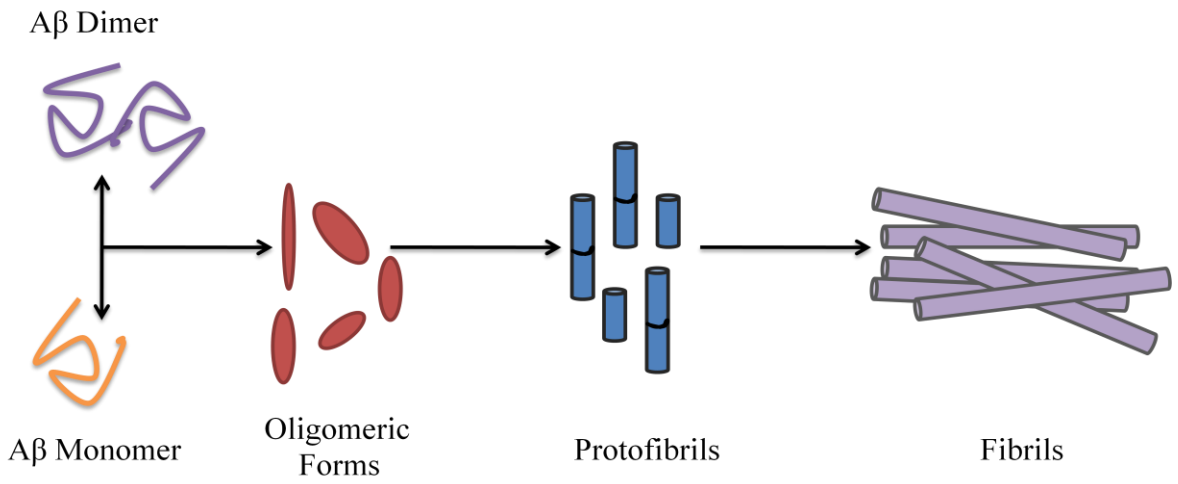

Figure 6: General model for Aβ aggregation

in other, unstructured aggregates called diffuse amyloid or preamyloid are formed. The preamyloid species exists in an amorphous form and is non-toxic to the neuronal cells. It is hypothesized that Aβ takes the ordered β-sheet pathway only when levels of total Aβ are above 10μM in the brain. This hypothesis explains why normal healthy brains do not develop AD like symptoms despite having Aβ present in them.

2.2.2.7. Structures of Aggregated Aβ

Elucidating the exact structure of aggregated Aβ could prove extremely useful in designing effective inhibitors for prevention of AD. However, previous studies have established that Aβ adopts different conformations depending on the environmental conditions (Nerelius, Johansson et al. 2009). The first 16 residues of Aβ are largely hydrophilic and the remaining residues form a part of a largely hydrophobic domain. Residues 12-23 have been identified as the self recognition sites for formation of dimers and higher oligomers. It is also posited that the hydrophobic stretch at residues 17-21 is critical in the formation of fibrillar structure (Ghanta, Shen et al. 1996). The exact structure of monomeric Aβ in solution is still uncertain as the psychological environment is difficult to achieve under laboratory conditions. Aqueous Aβ(1-40) was analyzed using Circular Dichroism and the result showed a mixed coil, β-turn, β-sheet and α-helical content for Aβ structure. Also, other groups observed a high β-sheet content at the air-water interface (Schladitz, Vieira et al. 1999), whereas in sodium dodecyl sulphate (SDS) micelles and later in 40% trifluoroethanol, monomeric Aβ showed an extensive α-helical content (Coles, Bicknell et al. 1998). However, the exact structure remains debated and it is postulated that Aβ adopts α-helical conformation in organic solvents whereas in aqueous buffers or water it is predominantly β-sheet (Serpell 2000).

Intermolecular β-sheet structure of the Aβ peptide fibrils was confirmed by a variety of techniques such as electron microscopy, Fourier transform infrared spectroscopy (FTIR), and CD (Serpell 2000; Irie, Murakami et al. 2005). The β-sheets are composed of cross-β-strands which are perpendicular to the axis of the fibril and intermolecular hydrogen bonding occuring parallel to its axis. Tjernberg et al. suggested that the smallest fibril forming sequence was Aβ (14-23) and this was the core of the Aβ fibril (Tjernberg, Callaway et al. 1999). It is proposed that residues 16-23 have a high propensity to form a β-sheet structure and residues 11-24 are implicated in α-helix to β-sheet conversion (Serpell 2000). One study indicated the presence of anti-parallel β-sheets in the Aβ fibrils and a turn at positions 25-28 due to the presence of the amide-I band of the infrared absorption spectra. On the other hand, structural studies on Aβ(10-35) using solid-state NMR have established a parallel β-sheet structure. This has led to a conclusion that Aβ fibrils can adopt both parallel and anti-parallel structure depending on the sequences and composition of the amino acid residues (Irie, Murakami et al. 2005). By the use of solid-sate NMR, it was suggested that fibrils made from different lengths of Aβ peptide were the same (Antzutkin, Leapman et al. 2002). In another study, it was proposed that the first 10 residues of Aβ(1-40) are structurally disordered. Petkova et al. presented a model in which residues 12-24 and 30-40 formed the β-sheet structure. The two β-sheets are in contact through side chain-side chain interactions with residues 25-29 forming the bend of the peptide (Petkova, Ishii et al. 2002). In the case of Aβ(1-40) fibrils, the side-chain interactions are intramolecular, whereas for Aβ(1-42) fibrils the upper layer sheet is displaced relative to the lower sheet so that the two β strands of Aβ molecule (*i*) form intermolecular side-chain interactions with the strands of molecules (*i+1*) and (*i-1*) respectively (Nerelius, Johansson et al. 2009). Many models have been proposed having different configurations, number of turns and pattern with each satisfying different constraints. Recent evidence suggests that the molecular structure

formed by the Aβ fibrils in-vitro depends on solvent composition, temperature, protein concentration, pH, ionic strength and on external mechanical forces such as agitation. Simple variation in these conditions can lead to the formation of fibrils with a completely different morphology (Pedersen and Otzen 2008). Also, direct structural measurements of Aβ fibrils are not possible due to the small quantities and the lack of isotopic labeling in-vivo. It was also demonstrated by Paravastu et al. that the molecular structure of Aβ fibrils seeded from AD brain fibrils were markedly different than those seeded with synthetic Aβ (Paravastu, Qahwash et al. 2009). Thus, even though in-vitro studies on Aβ fibrils have provided a plethora of knowledge, the exact structure of Aβ fibrils formed in-vivo remains uncertain.

2.2.2.8. Interactions between Aβ and Membranes

It is well documented from pathological, genetic and cell culture studies that Aβ 40/42 is the neurotoxic species in AD. Several investigators have postulated that the interaction of Aβ with cellular membranes may be the mechanism leading to cell death (McLaurin and Chakrabartty 1996; Choo-Smith and Surewicz 1997; McLaurin and Chakrabartty 1997; McLaurin, Franklin et al. 1998; Matsuzaki and Horikiri 1999; Kakio, Yano et al. 2004; Mandal and Pettegrew 2004; Verdier and Penke 2004; Chi, Frey et al. 2007). Aβ is known to interact with cell membranes and also with membranes of other subcellular components such as Golgi bodies, lysosomes and endoplasmic reticulum (Verdier and Penke 2004). Aβ in the aggregated form binds to neuronal membranes via hydrogen bonding and electrostatic interactions. It has been posited that Aβ(40/42) decreases the fluidity of the fatty acyl and head groups of the plasma, lysosomal and endosomal membranes whereas it increases the Golgi membrane fluidity (Waschuk, Elton et al. 2001). Investigations by Yanagisawa et al. showed the presence of monosialoganglioside GM1-bound Aβ (GM1-Aβ) in the brains of AD patients which is not

detected in non AD brains (Yanagisawa 2007). The GM1 ganglioside is a prominent lipid component of the cell membrane. They postulated that GM1-Aβ can act as a seed for Aβ polymerization leading to AD (Yanagisawa, Odaka et al. 1995; Yanagisawa and Ihara 1998). Terzi and co-workers showed that Aβ had higher binding affinity to the negatively charged phospholipids than zwitterionic and cationic lipids (Terzi, Holzemann et al. 1994; Terzi, Holzemann et al. 1997). It has been shown that cholesterol, gangliosides and membrane composition affects Aβ formation, Aβ aggregation and Aβ membrane association (Wang, Rymer et al. 2001). Some of the mechanisms that have been postulated to induce membrane-related toxicity are as follows: Strong physiological interactions of Aβ with membranes can lead to detrimental change in the fluidity of the membranes; interaction of Aβ with membranes leads to alterations in ion permeability, formation of ion channels, changes in intracellular Ca^{2+} levels leading to disturbed homeostasis and membrane depolarization; interaction of Aβ with membranes leads to disruption of neuronal homeostasis and loss of neuronal function (Verdier and Penke 2004; Chi, Frey et al. 2007). Thus, it is of utmost importance to understand the mechanisms and pathways through which Aβ-membrane association induces toxicity. There can be several mechanisms working together that may be the cause of increased Aβ or polymerized Aβ. However, we know for sure that Aβ interacts with the cell membrane where it binds to membrane lipids and this somehow leads to or contributes to toxicity. It is this theoretical bottleneck region that we are going to target in this thesis. For this, the role of gangliosides in AD must be reviewed.

2.2.2.9. Role of Gangliosides in AD Pathology

Lipid components such as glycerophospholipids, sphingolipids and cholesterol are the major components of cell membranes. Gangliosides are a type of glycosphingolipids containing

one or more sialic acid residues, with sialic acid being a generic term for *N*-acetyl- or *N*-glycoloyl-neuraminic acid (Schwarz and Futerman 1996). The hydrophilic characteristics of the big saccharidic headgroup and the hydrophobic characteristics of the double tailed sphingolipid called ceramide impart a strong amphiphilic nature to the gangliosides. The ceramide is composed by a long-chain amino alcohol, 2-amino-1,3-dihydroxy-octadec-4-ene, connected to a fatty acid by an amide linkage (Sonnino, Mauri et al. 2007). The sugar structure, content, sequence, bonding atoms in the oligosaccharide chain can vary along with the lipid moiety making gangliosides a very large family of compounds. Presence of sialic acid on the saccharidic headgroup differentiates gangliosides from neutral glycosphingolipids and sulfatides. The three main sialic acids known to be present in gangliosides are 5-*N*-acetyl-, 5-*N*-acetyl-9-*O*-acetyl and 5-*N*-glycolyl derivative. Due to their specific location, gangliosides are able to interact with a variety of biological entities such as glycoproteins, antibodies, peptides, hormones, growth factors etc. They are postulated to play an important role in the cell differentiation, biosignaling, inducing neuritogenesis and play a protective role in the case of neuronal injury (Lloyd and Furukawa 1998; Ariga, McDonald et al. 2008). GM1, GD1a, GD1b, and GT1b are the major gangliosides found in the human soma (Matsuzaki 2007). The structures of the major gangliosides are given in figure 7. It was observed that AD brains showed alterations in ganglioside levels and metabolism (Crino, Ullman et al. 1989; Ariga, McDonald et al. 2008) indicating that interactions between Aβ and membranes play a vital role in the pathology of AD. A novel ganglioside bound Aβ (GM1-Aβ) species was isolated from AD brain which was postulated to function as a seed for amyloid fibril formation (Yanagisawa, Odaka et al. 1995). Terzi et al. first reported the conformational change in Aβ from random coil to β-sheet after binding to negatively charged lipid vesicles (Terzi, Hölzemann et al. 1995; Ariga, McDonald et al. 2008). After that, many studies have reported the interaction between Aβ and gangliosides,

especially GM1, resulting in an altered secondary structure of the Aβ peptide (McLaurin and Chakrabartty 1996; Choo-Smith and Surewicz 1997; McLaurin, Franklin et al. 1998; Matsuzaki and Horikiri 1999; Kakio, Yano et al. 2004; Ariga, McDonald et al. 2008).

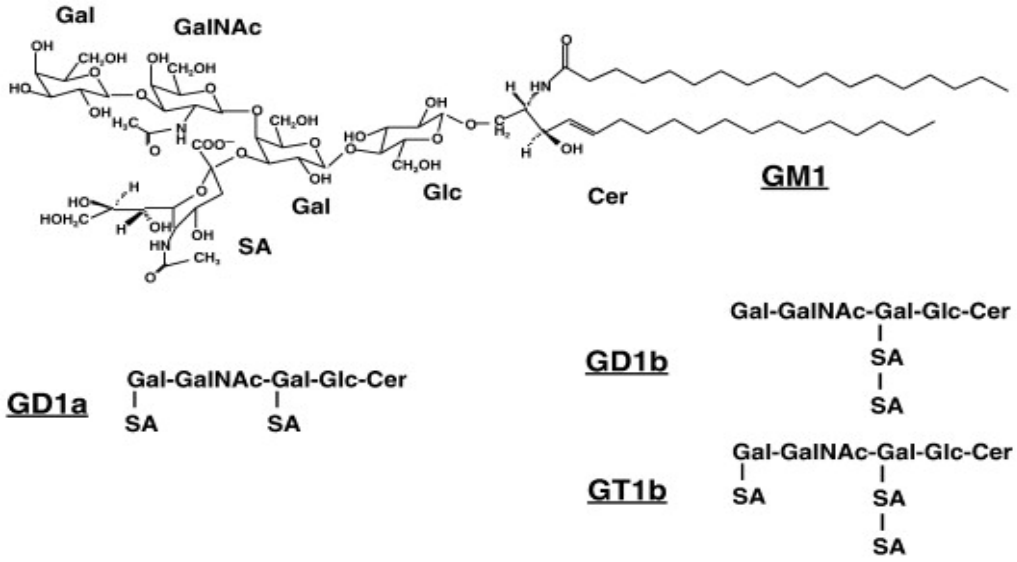

Figure 7: Chemical structures of major gangliosides present in neurons. Cer, ceramide; Glc, glucose; Gal, galactose; GalNAc, *N*-acetylgalactosamine; SA, sialic acid. (Matsuzaki 2007) Reprinted with permission from Elseveir Ltd.

In an important observation, Choo-Smith et al. showed that Aβ peptide interacts specifically with membrane gangliosides with affinities ranging from 10^{-6} to 10^{-7}M depending on the ganglioside sugar moiety. However, isolated oligosaccharide moiety on the ganglioside was not sufficient to induce the conformational change in Aβ peptide which indicated the role of the lipid component in the binding. They posited that the gangliosides function as high affinity receptors towards Aβ which leads to conformational changes from random coil to ordered β-sheet (Choo-Smith, Garzon-Rodriguez et al. 1997). Another important observation came from the studies of McLaurin and Chakrabartty, who reported that Aβ peptide disrupted acidic phospholipid membranes and the gangliosides induce Aβ40/42 to adopt a novel α/β conformation at neutral pH. They observed that the sialic acid moiety on the oligosaccharide chain was important for the inducing this disruption of the membranes. They speculated that gangliosides could sequester Aβ

and thereby prevent ordered β-sheet formation; alternatively, gangliosides may be involved in normal Aβ functioning and/or clearance (McLaurin and Chakrabartty 1996). McLaurin et al. suggested that the association with carbohydrate backbone was necessary along with the sialic acid for binding to Aβ. The study showed that the binding of Aβ(1-40) to mixed gangliosides or GM1 induced α-helical structure at pH 7.0 and β-sheet structure at pH 6.0. They posited that increasing the number of sialic acid residues on the carbohydrate backbone leads to increased net negative surface charge on the lipid vesicles which favors the formation of an ordered β-sheet structure and inhibits the α-helical structure (McLaurin, Franklin et al. 1998). This observation was further supported by the work of Matsuzaki and Horikiri, who suggested that Aβ(1-40) peptide binds more strongly to a ganglioside-rich domain in which the binding site was the sialic acid moiety, with the Aβ peptide adopting an antiparallel β-sheet lying parallel to the lipid bilayer (Matsuzaki and Horikiri 1999). In another work by Ariga et al. it was found that GM1 ganglioside had affinities in the following order of binding strengths: Aβ (1-42) > Aβ (40-1) > Aβ (1-40) > Aβ (1-38). Aβ-APP analogs had very low binding affinities for gangliosides. They also showed that Aβ(1-40) binds to a number of gangliosides with the following order of binding strength: GQ1bα > GT1aα > GQ1b > GT1b > GD3 > GD1a = GD1b > LM1 > GM1 > GM2 = GM3 > GM4. Their results suggested that an α2,3NeuAc residue on the neutral oligosaccharide core of gangliosides was required for binding along with the α2,6NeuAc residue linked to the GalNAc in the α-series (Ariga, Kobayashi et al. 2001). In another related study, researchers showed that Aβ has higher affinity towards clustered ganglioside GM1 and to gangliosides having higher number of sialic acid content, the formation of which is regulated by cholesterol content in the brain (Kakio, Nishimoto et al. 2001). In a study by Wang et al., reduction of cellular cholesterol and removal of cell surface sialic acids protected cells from Aβ toxicity, stressing the importance of surface sialic acids (Wang, Rymer et al. 2001). The clustering effect

of gangliosides is supported by the theory of lipid rafts. Lipid rafts are membrane microdomains that are enriched in cholesterol, sphingomyelin and sphingolipids (especially GM1 ganglioside) (Simons and Ikonen 1997). These lipid rafts are more ordered and tightly packed than surrounding bilayers, have certain proteins, signaling molecules clustered in it and have the ability to float freely in the cell membrane. Willliamson et al. demonstrated with NMR studies that interaction of ^{15}N-labeled Aβ(1-40) and Aβ(1-42) with GM1 micelles is localized to the N-terminal and His13 to Leu17 region of the peptide. They showed that the fibrillogenic seed nucleus involves an interaction with His13 with the sialic acid moiety of GM1 ganglioside. This indicated that Aβ binds to the carboxylic acid group on sialic acid via a positively charged amino acid residue. However, they observed no binding to the isolated pentasaccharide headgroup, suggesting the need for a polyanionic membrane like surface (Williamson, Suzuki et al. 2006).

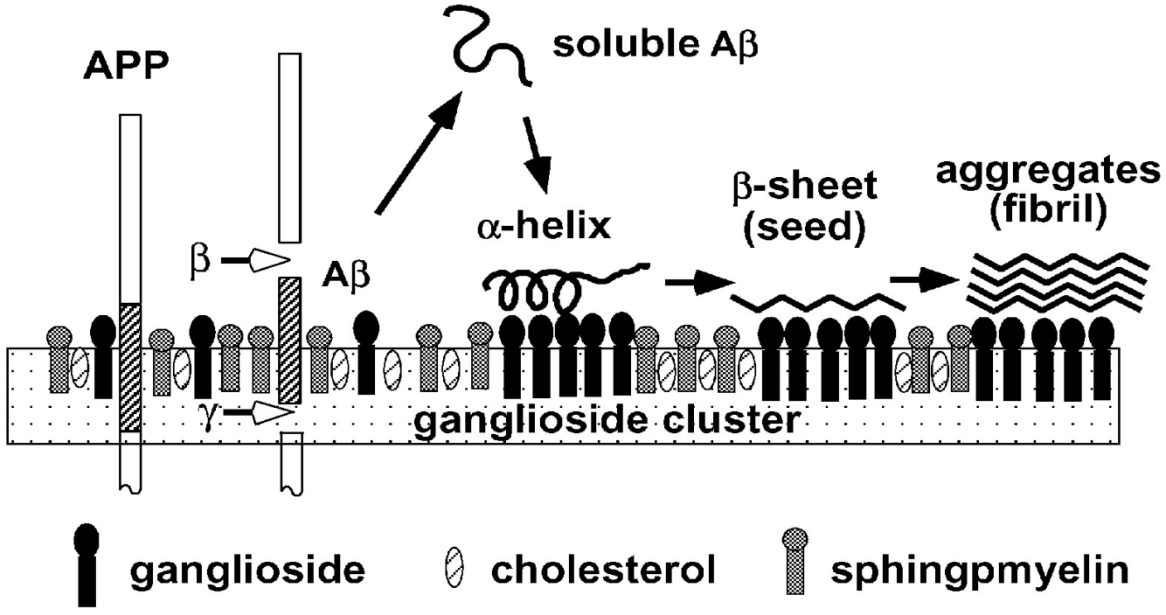

Figure 8: Hypothetical mechanism of ganglioside-mediated Aβ fibrillization. Enzymatic cleavage (γ- and β-secretase) of APP generates soluble Aβ in the lipid rafts composed of cholesterol and sphingolipids. Cholesterol mediates the formation of ganglioside clusters (especially GM1), Aβ binds to the clusters, forming a seed for further soluble amyloid deposition into ordered β-sheet form at higher peptide to ganglioside ratios (Ariga, McDonald et al. 2008). Reprinted with permission from Elseveir Ltd.

A number of studies have observed the accumulation of specific ganglioside bound Aβ complex in the AD brain (Yanagisawa, Odaka et al. 1995; Yanagisawa and Ihara 1998; Yanagisawa 2007; Ariga, McDonald et al. 2008). Other studies showed the interaction between gangliosides and Aβs peptides in neuronal cells leading to the amyloid fibril formation. All of these results point to the pivotal role of gangliosides especially GM1 in the pathogenesis of Alzheimer's Disease.

We can propose that the initial step in AD is ganglioside binding with the Aβ peptide, the peptide then undergoes self-association on the membrane surface by undergoing a conformational change from random coil to ordered β-sheet. This surface associated, ordered β sheet peptide then acts as a specific template ("seed") which causes additional soluble Aβ to form fibrils by β-sheet augmentation mechanism (Choo-Smith, Garzon-Rodriguez et al. 1997; Ariga, McDonald et al. 2008). [Please see (Ariga, McDonald et al. 2008) for excellent review]

2.3. Diagnosis of AD

The diagnosis rate of Alzheimer's disease is generally low as definite diagnosis of AD can only be made post-mortem after the analysis of neurofibrillary tangles and amyloid plaques (Craig-Schapiro, Fagan et al. ; Boss 2000; Blennow, de Leon et al. 2006). However, recent advances have led to the development of some clinical tests and laboratory markers which have shown limited success in diagnosis of AD. The criteria of the National Institute of Neurological and Communicative Disease and Stroke and the Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA) is most commonly used for the clinical diagnosis of AD (Blennow, de Leon et al. 2006; Minati, Edginton et al. 2009). These criteria's establish steps to exclude other dementias from AD and also to determine the presence of gradual onset with significant cognitive impairment. The results of the test will classify the diagnosis as definite

(clinical diagnosis with histology confirmation), probable (typical clinical syndrome without histology confirmation) or possible (atypical clinical features but no histology confirmation) AD (Cummings 2004). However, detailed neuropsychological assessment is needed to support the diagnosis so as to differentiate between AD and other types of dementias. The recent trend followed is to use these tests in conjunction with neuroimaging techniques as a source of supportive evidence.

In addition to these clinical tests, a number of molecular and biochemical markers are in development. An ideal biomarker for AD should be able to detect the fundamental feature of AD neuropathology, should be valid in confirmed AD cases, should be precise and reliable, simple to perform and inexpensive (The Ronald and Nancy Reagan Research Institute of the Alzheimer's Association and National Institute on Aging Working Group 1998). The proteolytic processing of transmembrane APP produces the Aβ peptide which is deposited as senile plaques. The measures of APP and its derivatives in the blood or CSF may be used as biomarkers for AD. Though earlier studies had supported this theory, recent investigations have reported inconsistent results (Craig-Schapiro, Fagan et al.). Approximately 60% of AD populations have at least one APOE ε4 allele and so genotyping of APOE allele is investigated as a biomarker of AD. However, late studies established that this biomarker had limited predictive power (McConnell, Sanders et al. 1999). Assaying Aβ(1-42) levels, Aβ(1-40) levels, total Aβ levels, tau protein levels and the combination of different ratios of the above mentioned peptides in the CSF have been investigated by many researchers with limited success (Craig-Schapiro, Fagan et al.). As AD brains demonstrate oxidative stress damage, the role of isoprostanes, which are the end-products of lipid peroxidation have been investigated as potential biomarkers. AD brains also show the presence of inflammatory processes and markers which can particularly identify such

processes have been studied but have inconsistent results (Craig-Schapiro, Fagan et al.). Most of the discrepancies in the results seem to arise due to heterogeneity of the study samples, methods used for assaying, environmental exposures etc.

Neuroimaging techniques are being developed to detect the changes in brains of patients associated with AD. Studies have indicated that the first degenerative changes in the brain occur in the medial temporal lobe, including the hippocampus and entorhinal cortex (Scheltens, Fox et al. 2002). Computed tomography (CT) and magnetic resonance imaging (MRI) have been applied to study AD brains. MRI can reveal atrophy of the hippocampus and of the entorhinal cortex and can also distinguish AD patients from controls by volumetric analysis. Functional MRI is another neuroimaging technique that has been applied in the diagnosis of AD where it can reveal abnormalities in brain activation (Craig-Schapiro, Fagan et al.). Positron emission tomography (PET) is another technique that has been applied to measure glucose metabolism from cortical areas using ^{18}F-fluoro-deoxy-glucose (^{18}F-FDG) marker as glucose metabolism is reduced as a result of synaptic loss and metabolic dysfunction in AD (Minati, Edginton et al. 2009). Recent research is aimed at developing ligands that have specific affinity towards amyloid fibrils, NFTs and activated microglia. ^{18}F-1,1-dicyano-2-[6-(dimethylamino)-2-naphtalenyl] propene (^{18}F-FDDNP), *N*-methyl [^{11}C] 2-(4'-methylaminophenyl)-6-hydroxybenzothioasole (commonly known as Pittsburgh compound B, ^{11}C-PIB), 4-*N*-methylamino-4'- hydroxystilbene [^{11}C] (^{11}C-SB13) and another known as 2-(2-[2-dimethylaminothiazol-5-yl]-ethenyl)-6-(2-[fluoro]ethoxy)- benzoxazole (^{11}C-BF-227) are some novel radiotracers being investigated as possible ligands for diagnosis of AD (Nordberg 2007; Minati, Edginton et al. 2009).

Thus, the search for a reliable biomarker of AD is still ongoing. Limited sample size, differences in individual samples such as age, gender, ethnicity, differences in protocols,

methods and analysis are some of the issues that impede reliable biomarker development. Researches now postulate that given the multifaceted nature of AD, instead of a single biomarker, a set of biomarkers may give reliable accuracy and appropriate sensitivity in diagnosis of AD.

2.4. Current and Emerging Therapeutic Approaches Targeting Aβ

2.4.1. Drugs for AD

Currently available treatments for AD are merely symptom alleviating, providing temporary cognitive improvement and deferred decline. However, they show very little to no evidence of slowing disease progression or curing AD (Klafki, Staufenbiel et al. 2006). Table 2 lists the drugs currently approved by the Food and Drug Administration (FDA).

Most of the drugs approved today are cholinesterase inhibitors. The enzyme cholinesterase is responsible for the degradation of acetylcholine, which is released into the synaptic cleft after the firing of the synapses from one neuron to another (Klafki, Staufenbiel et al. 2006). As neurons are under attack in AD, they produce less acetylcholine and hence, inhibition of its destruction causing enzyme makes more neurotransmitters available for communication between neurons. Galantamine, Donepezil and Rivastigmine are the 3 inhibitors of cholinesterase available in the market today and are approved for mild to moderate treatment of AD (Blennow, de Leon et al. 2006; Klafki, Staufenbiel et al. 2006; Minati, Edginton et al. 2009; Roland Jakob-Roetne 2009). Galantamine and Donepezil selectively inhibit acetylcholinesterase hydrolysis in the brain, while Rivastigmine in addition to cholinesterases also inhibits butyrylcholinesterase, which has a similar role to that of cholinesterase. Based on their mechanism of action it is evident that these medications only temporarily mitigate symptoms and are not expected to change the course of AD (Blennow, de Leon et al. 2006).

Table 2: Characteristics of Drugs Approved for AD (Blennow, de Leon et al. 2006; Mandavilli 2006; Lleo 2007).

	Donepezil	Galantamine	Rivastigmine	Memantine
Manuf-acturer	Eisai Inc. / Pfizer, NY	Ortho-McNeil Neurologics Inc.	Novartis Pharmaceuticals Corporation	Forest Pharmaceuticals
Launched	1997	2001	2000	2003
Indication	Mild to Moderate AD	Mild to Moderate AD	Mild to Moderate AD	Moderate to Severe AD
Mode of Action	Selective Cholinesterase inhibition	Selective Cholinesterase inhibition	Selective Cholinesterase and Bututylcholinesterase inhibition	Non-Competitive NMDA-receptor antagonist
Half-Life	Long (70h)	Short (7-8h)	Very Short (1h)	Long (60-100h)
Major Side Effects	Nausea, vomiting, diarrhea, fatigue, insomnia, muscle cramps, anorexia	Nausea, vomiting, diarrhea, anorexia, weight loss	Nausea, vomiting, loss of appetite, indigestion, weakness, dizziness, diarrhea, stomach pain	Dizziness, constipation, confusion, headache

All the three drugs reported positive effects in several randomized, double-blind, placebo-controlled studies however, a direct comparison of these three cholinesterase inhibitors has not been done. Another drug available is Memantine, an NMDA (*N*-methyl-*D*-aspatarte) -receptor, which is approved for the treatment of moderate to severe AD. In AD, it is observed that NMDA glutamate receptors are overactivated which leads to disturbed calcium homeostatis causing neurodegeneration. Memantine is a non-competitive NMDA-receptor antagonist with moderate affinity that appears to be able to protect neurons while leaving physiological NMDA-receptor

activation unaffected (Klafki, Staufenbiel et al. 2006). Several other potential NMDA receptors are in active phases of development. Some researchers postulate that the use of Memantine with cholinesterase inhibitors might be a viable approach in treating AD. In a randomized, double-blind, placebo-controlled clinical trial of patients with moderate to severe AD, the combination therapy showed a statistical significant benefit over monotherapy of Donepezil, with regard to the measures of cognitive function, activities of daily living, and behavior (Klafki, Staufenbiel et al. 2006). However, this theory needs to be investigated further.

2.4.2. Immunotherapy for AD

Immunotherapy is an emerging and promising approach because it promotes the possibility of peripheral treatment of Aβ that eliminates the need to design molecules that can cross the blood-brain barrier (BBB). The idea behind immunotherapy is that by decreasing the Aβ levels in the blood, more Aβ can be removed from the brain. The use of Aβ immunotherapy was first reported by Schenk and co-workers from the study in APP transgenic mice, wherein active immunization with fibrillar Aβ attenuated Aβ deposition and improved behavior (Schenk, Barbour et al. 1999; Klafki, Staufenbiel et al. 2006). Similar results were obtained by the use of passive immunization with antibodies against Aβ (Bard, Cannon et al. 2000). This led to the clinical trials on mild AD patients with vaccine AN1792, composed of preaggregated Aβ(1-42). However, in phase II, it was found that 6% of the vaccinated cases had developed aseptic meningoencephalitis and the trail was discontinued (Gilman, Koller et al. 2005). The researchers attributed this side-effect due to the T-cell response against the mid-terminal and C-terminal part of the peptide (Blennow, de Leon et al. 2006; Roland Jakob-Roetne 2009). Still, refined forms of active immunization are considered as a viable option and some clinical trials are in Phase I (Brody and Holtzman 2008). Researchers have also focused on the development of passive

vaccinations for the treatment of AD. Several trials with passive immunization are underway with selective monoclonal antibodies which have been shown to decrease Aβ plaque pathology and reduce behavioral impairments in transgenic mice (Minati, Edginton et al. 2009). Phase III trials are under way for an anti-Aβ antibody named Bapineuzumab, which has shown affinity for both soluble and insoluble Aβ. One phase II and two phase I trials are also underway for other antibodies (Brody and Holtzman 2008). Thus, active/passive immunization or vaccination can prove to be a viable option for the treatment of Aβ. A very good strategy in immunotherapy is to develop an Aβ sequestering molecule that does not elicit an immune response.

2.4.3. Inhibition/ Modulation of Secretases

According to the amyloid cascade hypothesis, the production of Aβ is the root-cause of AD. Assuming this to be true, the inhibition/modulation of secretases would be the cleanest approach which would remove monomeric Aβ, therefore preventing the production of oligomers and fibrils. Thus, up-regulation of α-secretase, down-regulation/inhibition of β-secretase, and inhibition/ modulation of γ-secretase are some of the potentially viable approaches that are being investigated currently.

γ-Secretase inhibitors can reduce Aβ synthesis which can further inhibit downstream cascade of events. DAPT, LY450139 dihydrate, MRK-560 and BMS-299897 are some of the γ-secretase inhibitors that have shown marked reduction in Aβ levels in brains, CSF and plasma in transgenic mice (Klafki, Staufenbiel et al. 2006; Roland Jakob-Roetne 2009). However, it was found that γ-secretase cleaves substrates other than APP such as Notch (Minati, Edginton et al. 2009). Thus, inhibition of γ-secretase can have adverse effects. Hence, modulating the activity of γ-secretase to produce less Aβ is a more viable strategy. Recently, a γ-secretase inhibitor was developed that was able to inhibit Aβ production without affecting Notch signaling (Scarpini,

Schelterns et al. 2003). Another γ-secretase modulator that has entered phase III study is R-flurbiprofen, which is believed to lower the production of the more toxic Aβ(1-42) by shifting the cleavage of APP from producing Aβ(1-42) to other shorter, less toxic peptide fragments (Seow and Gauthier 2007). Several other γ-secretase modulators are currently being developed or undergoing phase I trials (Minati, Edginton et al. 2009). Also, β-secretase appears to be the perfect therapeutic target as it represents the first step in Aβ production. Studies on β-secretase (BACE1) knockout mice revealed very small quantities of Aβ thereby establishing BACE1 as the primary β-secretase enzyme acting in-vivo (Scarpini, Schelterns et al. 2003; Klafki, Staufenbiel et al. 2006). However, the physiological roles of BACE1 and its homologue BACE2 are unknown even though they are expressed throughout the body. The complete inhibition of β-secretase have shown potentially deleterious effects in mice. It was observed that β-secretase can also act on other non-APP substrates (Barten and Albright 2008; Cole and Vassar 2008). It has been difficult to develop potent brain penetrant BACE1 inhibitors as it was observed that most of BACE inhibitors showed nanomolar binding affinities in cell free assays but were unsuitable for in-vivo experiments (Roland Jakob-Roetne 2009). Recently, it was announced that a potent BACE1 inhibitor, named CTS-21166 (Cole and Vassar 2008) was safe and well-tolerated in Phase I study. In AD, it is believed that β and α-secretase compete for the APP substrate and there exists a balance between the two activities.

Progress in developing efficient inhibitors and modulators of secretases has been impeded as most of these secretases have not been fully identified and understood. Their psychological roles are unknown. Not everything is known about the different substrates they attack and it is believed that most secretases attack more than one substrate. Thus, there is a need for developing selective and highly targeted drugs that can only inhibit or modulate the APP

cleavage process. Also, the compounds developed should be capable of crossing the blood-brain barrier. Such constraints make the development of specific drugs a challenging task.

2.4.4. Inhibiting the Aggregation of Aβ

Preventing the aggregation of Aβ, thereby preventing the formation of presumed toxic oligomerates and fibrils by specifically binding molecules is another promising approach for the treatment of AD. Alzhemed (3-amino-1-propanesulphonic acid), a small molecule developed by Neurochem Inc., has been shown to inhibit the interaction of Aβ with glycosaminoglycans thereby inhibiting formation of Aβ aggregates. Clioquinol (PBT-1) developed by Prana Biotechnology, has shown good results in reducing Aβ deposition in APP transgenic mice by binding to zinc and copper, which are postulated to be involved in Aβ aggregation process (Scarpini, Schelterns et al. 2003). Also, another metal chelating agent, PBT-2 is in phase II trials (Barten and Albright 2008). Another approach is to design inhibitors based on histological dyes used to characterize amyloid in-vitro and in-vivo. A number of polyphenols such as Curcumin, Catechins, Gingo Biloba are also being investigates are potent inhibitors of Aβ aggregation and to prevent neurotoxicity (Hawkes, Ng et al. 2009).

Compared to small molecules, several peptide based therapeutic strategies are under investigation, as they are thought to be more effective as they can interact with the extended regions of Aβ. Tjernberg et a. reported that a pentapeptide KLVFF of Aβ(16-20) binds to and disrupts fibrils formation (Tjernberg, Naslund et al. 1996). Ghanta et al. reported a prototype inhibitor composed of residues 15-25 of the Aβ peptide linked to an oligosine disrupting element (Ghanta, Shen et al. 1996). The use of the recognition element helps in specificity whereas the disrupting element interferes with Aβ aggregation pathway. Selective substitution of proline at key positions on a peptide homologous to the central 17-21 regions of Aβ was shown to convert

Aβ fibrils to amorphous aggregates and inhibit toxicity in-vitro and in-vivo (Soto, Sigurdsson et al. 1998). The use of *N*-methylated peptides is another promising approach which is known to lock the residues into a β-conformation. *N*-methylated peptides function by binding to the face of the aggregating peptide through the amide -NH groups at the outer edge of the β-sheet, effectively blocking intermolecular hydrogen bonding, thus preventing aggregation and toxicity (Hawkes, Ng et al. 2009).

However, most of these strategies are under development and their beneficial effects on AD patients are still not clear. Another major problem is that the most toxic species of Aβ has not been identified. Also, determining the correct chain of events in AD development is challenging and this is another major hurdle in the development of a specific Aβ inhibiting molecule.

2.4.5. Drug Based on Epidemiology

Epidemiological studies have observed the protective effect of different types of drugs and supplements on AD patients. One such class of drugs being investigated are anti-inflammatory drugs as inflammation type characteristics are observed in the immediate vicinity of plaques. Hence, it is believed that use of anti-inflammatory drugs may have preventive effect in the development of AD either by the inhibition of cyclo-oxygenase (COX-1 or COX-2) or by direct action on γ-secretase (Blennow, de Leon et al. 2006; Klafki, Staufenbiel et al. 2006). However, clinical trials on drugs such as Prednisone, Hydroxychloroquine, Naproxen, Celecoxib and Rofecoxib were negative (Barten and Albright 2008). The use of cholesterol reducing drugs (statins) is suggested as a viable treatment option for AD as in-vivo and in-vitro studies showed altered levels of APP and Aβ along with cholesterol levels (Eckert, Muller et al. 2007). However, clinical trials of these drugs have showed ambiguous results (Klafki, Staufenbiel et al.

2006; Eckert, Muller et al. 2007). One of the reasons may be that the exact role of cholesterol in AD is unknown. Dietary intake of anti-oxidants such as Vitamin E has shown beneficial results as oxidative stress is lessened in an AD brain. However, most of these strategies target post-cell damage and are therefore not optimal disease modifying agents.

2.4.6. Novel Aβ Sequestering Agents

In the earlier sections, we have already reviewed the interaction between Aβ, gangliosides and the neuronal membrane. We know that Aβ interacts with cells via binding to surface glycolipids and glycoproteins, and that the affinity of this interaction increases when the gangliosides or sialic acid molecules on the cell surface are clustered (Choo-Smith, Garzon-Rodriguez et al. 1997; McLaurin and Chakrabartty 1997; Ariga, Kobayashi et al. 2001; Kakio, Nishimoto et al. 2001; Kakio, Yano et al. 2004; Patel, Henry et al. 2006; Williamson, Suzuki et al. 2006; Patel, Henry et al. 2007). Based on these observations, the approach is to design membrane mimics that would reproduce the clustered sialic acid structure of the cell surface thus successfully competing with the cell surface for Aβ binding. Then, Aβ would have higher affinity towards the mimic, binding it instead of the cell membrane, and thus sequestering the Aβ, thereby reducing its neurotoxicity (Bard, Cannon et al. 2000; Patel, Henry et al. 2006; Patel, Henry et al. 2007). Such a strategy has already been applied with good results to many biological systems such as the prevention of influenza viral adhesion and infection both in-vitro and in-vivo (Reuter, Myc et al. 1999; Landers, Cao et al. 2002; Makimura, Watanabe et al. 2006; Cowan, Coté et al. 2008; Umemura, Itoh et al. 2008). Patel et al. synthesized sialic acid conjugated dendrimers which were more effective than unconjugated dendrimers alone at reducing Aβ toxicity. The reported binding affinities for Aβ to gangliosides in various literatures are in the order of 10^{-6}M (Choo-Smith, Garzon-Rodriguez et al. 1997; Choo-Smith and Surewicz 1997;

Ariga, Kobayashi et al. 2001) whereas the observed binding affinities for Aβ to sialic acid conjugated dendrimers were on the order of 10^{-7} to 10^{-9} M. The improved affinity was attributed to the clustering of sialic acids on dendrimers, or the combined effect of electrostatic interactions of Aβ with the dendrimer backbone and the interaction of Aβ with the surface sialic acids on the dendrimer (Patel, Henry et al. 2006). However, the dendrimer backbone itself was toxic to the cells, leading to lower viability. This observation was in agreement with that observed in literature for dendrimer toxicity (Reuter, Myc et al. 1999; Mishra, Gupta et al. 2009). Also, it is possible that due to the rigid structure of the dendrimer used, the star burst type, it could have partly reduced the binding of labeled sialic acid to specific Aβ sites. In another related work by Patel et al., the difference in Aβ attenuation using physiologically relevant attachment (via anomeric hydroxyl) versus non-physiologically attached (carboxyl attached) chemistry of dendrimers was investigated. They found that though physiologically attached dendrimers attenuated Aβ toxicity at lower concentrations than non-physiologically attached dendrimers, there was no significant improvement in the binding affinities (Patel 2007). Furthermore, they postulated that, greater Aβ toxicity attenuation could be achieved by the use of a less highly charged polymer backbone and longer spacer between the charged polymer and sialic acid (Patel, Henry et al. 2007). In the work by Cowan et al., use of photocrosslinked sialic acid containing oligosaccharides 3'–sialyl-*N*-acetyllactosamine (3'SLN) and disialyllacto-*N*-tetraose (DSLNT), showed almost complete attenuation of toxicity but at very high polymer concentration. Their results suggested that the mechanism of toxicity attenuation of Aβ might not be direct competition for Aβ binding and that, better attenuation of toxicity could be achieved at lower concentrations by increasing the valency of sialic acids on the polymers (Cowan, Coté et al. 2008). All these observations support the fact that multivalency can increase the binding affinity of a ligand to receptors (Cowan, Patel et al. 2009). By the use of mathematical models, Cowan et

al. showed that the closest qualitative explanation for the membrane mimic attenuating toxicity was that the sialic acid mimic bound to Aβ, the Aβ-mimic complex was still toxic to the cells, but with a reduced toxicity compared to Aβ alone. It is predicted that at physiological ionic strengths, electrostatic effects are likely to play a role in sialic acid polymer toxicity attenuation of Aβ only for very highly charged polymers (Cowan, Patel et al. 2009).

All these observations certainly indicate that there are yet to be understood mechanisms that are possibly playing a role in attenuating Aβ toxicity such as the possibility of different –R group sugars that have better properties. Since, a number of studies have highlighted the role of multivalent sialic acids in attenuating toxicity, it will be crucial to investigate how multivalency affects Aβ binding. In addition, the effect of different backbones on the Aβ needs to be investigated as backbone toxicity can significantly affect viability. This leaves the field wide open for the design of better membrane mimicking compounds that can attenuate toxicity at even lower concentration with higher affinity.

Towards this goal, there is a need for a suitable backbone structure that is biocompatible, flexible, non-toxic and easy to label among other things. Chitosan is an aminopolysaccharide that seems ideal as a backbone for sialic acid labeling. We will aim to synthesize sialic acid labeled chitosan by the use of a suitable cross-linker and test the efficiency of this complex to attenuate Aβ toxicity.

2.5. Chitosan

Chitosan is a natural amino-polysaccharide comprising copolymers of D-glucosamine and N-acetyl-*D*-glucosamine linked together by β(1-4) glycosidic bonds (Pillai, Paul et al. 2009). It is derived by partial deacetylation of chitin from crustacean shells. The content of glucosamine in chitosan is called the Degree of Deacetylation (DD), which affects its solubility. In chitosan,

the DD ranges from 40% to 98% and the molecular weight ranges between 300Da to over 1000kDa depending on the source and method of preparation (Kumar, Muzzarelli et al. 2004; Kim, Seo et al. 2008). The oral mean lethal dose of chitosan in mice was found to be in excess of 16g/day/kg, which is higher than sucrose (Raafat, von Bargen et al. 2008).

Chitosan is normally insoluble in aqueous solutions above pH 7 and in dilute acids (pH < 6), the amino groups on glucosamine become protonated (pKa value of 6.3) facilitating solubility of chitosan, making it a cationic polyelectrolyte. Generally, chitosan has three types of reactive functional groups, an amino group as well as both primary and secondary hydroxyl groups at the C2, C3 and C6 positions, respectively, which allow modification of chitosan for various applications (Kim, Seo et al. 2008).

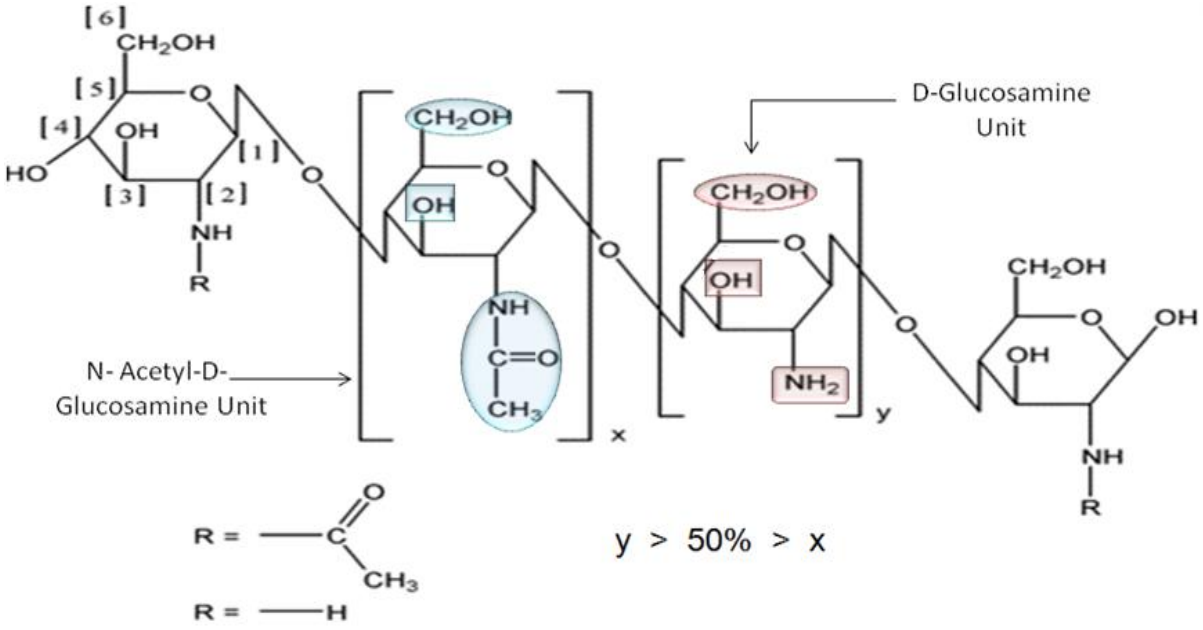

Figure 9: Structure and possible reaction sites in chitosan (Pillai, Paul et al. 2009)

. The amino functionality gives rise to chemical reactions such as acetylation, amide formation, quaternization, reactions with ketones and aldehydes, alkylation, grafting, chelation of metals etc. Much work has been reported on chemical modifications of chitosan and have been

reviewed extensively (Ravi Kumar 2000; Kumar, Muzzarelli et al. 2004; Muzzarelli and Muzzarelli 2005; Kim, Seo et al. 2008; Pillai, Paul et al. 2009). Chitosan and its derivatives have been shown to have several interesting properties such as biocompatibility, biodegradability to harmless products, physiological inertness, remarkable affinity to proteins, nontoxicity, antibacterial, hemostatic, fungistatic, antitumoral, anti-acid, non-allergic, antiviral and anticholesteremic properties (Kumar, Muzzarelli et al. 2004; Muzzarelli and Muzzarelli 2005; Kim, Seo et al. 2008; Pillai, Paul et al. 2009). Such unique properties of chitosan makes it a very interesting topic of research with a host of applications (Ravi Kumar 2000; Kumar, Muzzarelli et al. 2004; Muzzarelli and Muzzarelli 2005; Kim, Seo et al. 2008; Pillai, Paul et al. 2009).

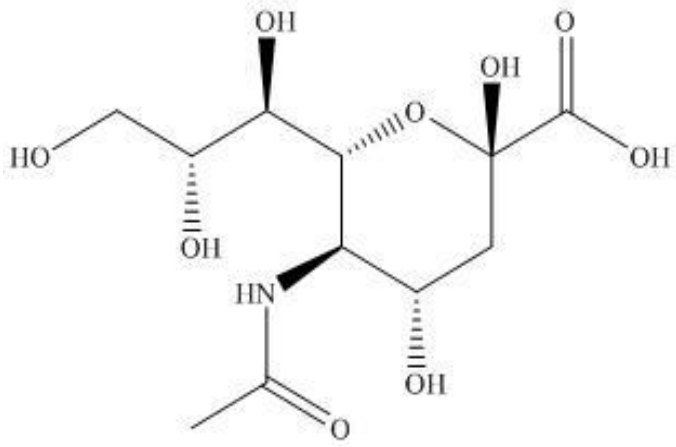

Figure 10: Structure of sialic acid (*N*-acetylneuraminic acid)

We envisioned the synthesis of sialic acid labeled chitosan that can mimic the sialic acid structure present on the cell membrane. For this reason, a suitable conjugation chemistry should be investigated that can attach sialic acid to chitosan with control over the degree of labeling. Looking at the structure of sialic acid, it is seen that –COOH group is present at position C-1. A number of chemistry methods have been developed that can couple $–NH_2$ group to a –COOH group resulting in amide linkage. A conjugation chemistry used is reviewed in the next section.

2.6. Conjugation Chemistry Using EDC with Sulfo-NHS

Crosslinker reagents are commonly used to couple two molecules together. Homobifunctional crosslinkers (e.g. Imidoesters to couple amines) are used when the two crosslinking molecules have the same functional groups and heterobifunctional crosslinkers (eg. carbodiimides to from amide linkages) are used when the two targeted molecules have different functional groups. In using conjugation chemistry, the length of the spacer arm or bridge is an important consideration as it can affect the steric interactions and affinity between linked molecules (Mattson, Conklin et al. 1993).

EDC or 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride is a zero-length, water soluble crosslinker used commonly to couple carboxylic acids with primary amines. EDC first reacts with the carboxyl groups to form a highly reactive, *O*-acylisourea intermediate. This active species then reacts with an amino group to form an amide bond by release of an isourea derivative as by-product. However, the intermediate is unstable in aqueous solutions and therefore, a two-step conjugation procedure is preferred using *N*-hydroxysuccinimide (NHS) or *N*-hydroxy-sulfosuccinimide (Sulfo-NHS) for stabilization. Sulfo-NHS esters are water soluble hydrophilic active groups that react rapidly with amino groups on target molecules. This two-step procedure of using EDC and Sulfo-NHS has advantages such as enhanced coupling efficiency, slow hydrolysis in water, extension of the half-life of activated carboxylate from seconds to hours and increased stability in coupling. Thus, in the first step, EDC reacts with the carboxyl group on first target compound forming the unstable *O*-acylisourea ester intermediate. Sulfo-NHS, added at the same time as EDC, reacts with the unstable intermediate to form semi-stable amine reactive NHS ester with improved stability. In the second step, the amino group containing molecules are added. In the presence of amino groups that can attack the carbonyl

group of the ester, the *N*-hydroxy-sulfosuccinimide group rapidly leaves, creating a stable amide linkage with the amine molecule. Failure to react with an amine results in hydrolysis of the intermediate, regeneration of the carboxyl groups and release of *N*-substituted urea (Grabarek and Gergely 1990; Mattson, Conklin et al. 1993; Hermanson 2008). The mechanism of EDC chemistry is given in figure11.

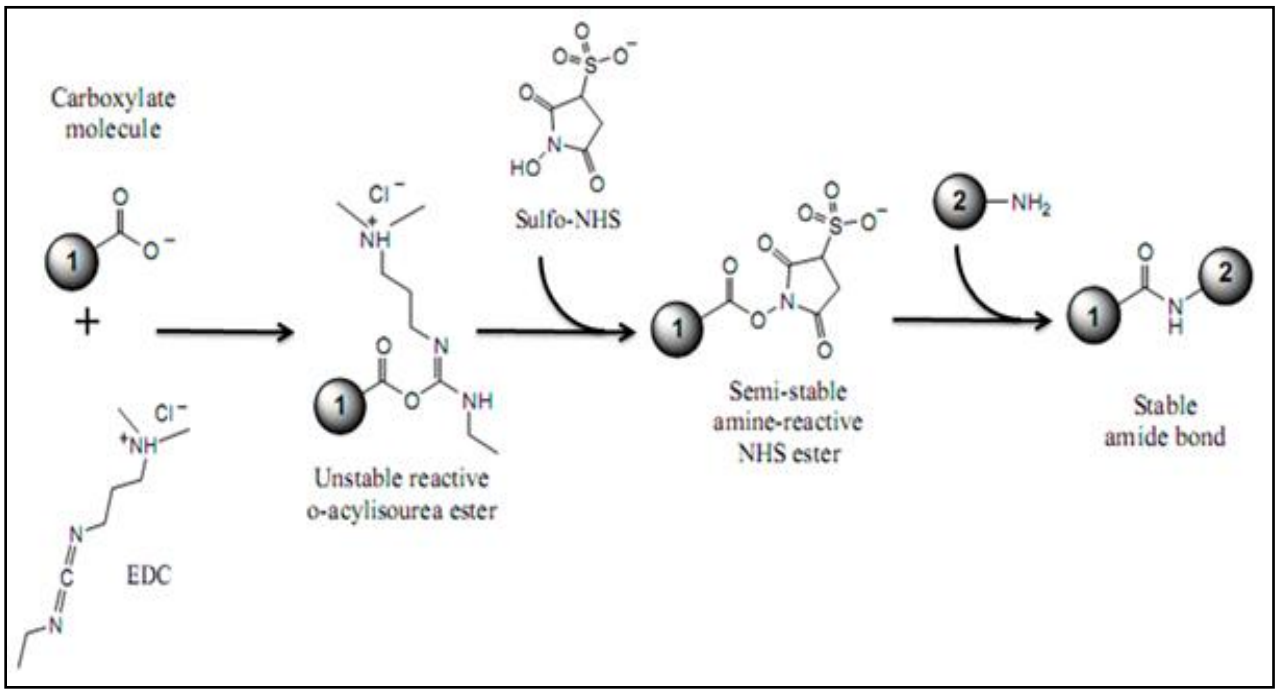

Figure 11: Mechanism using EDC and Sulfo-NHS to couple carboxylate containing molecules with amine containing molecules showing intermediate steps (Hermanson 2008) (http://www.piercenet.com/products/browse.cfm?fldID=02040114)

The EDC coupling reaction is dependent of temperature, pH and buffer composition (Mattson, Conklin et al. 1993). Studies have found that reactions with EDC and Sulfo-NHS are most efficient at pH between 4.7 and 6. At low pH, MES buffer (2-[morpholino]ethanesulfonic acid) at 0.1M is recommended whereas for neutral pH reactions, phosphate buffers can be used. Amine or carboxylate containing buffers can interfere with the EDC chemical pathway and hence should be avoided. For the two step conjugation process, the first reaction is usually performed in MES buffer (or other non-amine, non-carboxylate buffer) at pH 5.0-6.0. After

activation is complete, the pH is raised by using phosphate buffer (or other non-amine buffer) to 7.0-7.5 immediately before addition of amine containing compound (Ref: NHS, Sulfo-NHS product information sheet, Pierce Biotechnology, IL, USA) (Grabarek and Gergely 1990; Hermanson 2008). Thus, EDC chemistry is extensively used to couple two proteins, haptens to carrier proteins, surface molecule attachment and a host of other applications. Practically, any two molecules having a carboxyl group and amine group can be conjugated by this chemistry. The disadvantages of this chemistry include unwanted polymerization, precipitation of conjugating molecules, hydrolysis and unwanted side reactions in presence of certain compounds.

3. SYNTHESIS OF SIALIC ACID LABELED CHITOSAN

3.1. Experimental Procedures

3.1.1. Materials

Chitosan powder (MW~15000, DD~84%) was purchased from Polysciences Inc. (Warrington, PA). 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and *N*-hydroxysuccinimide (Sulfo-NHS) were purchased from Pierce Biotechnology (Rockford, IL). Ultrafilteration membranes were purchased from Millipore (Billerica, MA). Sialic acid (*N*-Acetylneuraminic acid), chemicals for Warren assay and all other reagents were purchased from Sigma-Aldrich (St. Louis, MO).

3.1.2. Synthesis and Purification of Sialic Acid Labeled Chitosan

Sialic acid was conjugated with chitosan using EDC chemistry following the manufacturer's suggested protocol with minor modifications. Chitosan (MW~ 15000, DD~ 84%) at concentration of 8 mg/ml, was dissolved in 1X Phosphate Buffer Solution (PBS) and 5% HCl solution. Acidic medium was necessary to dissolve chitosan and 8 mg/ml of chitosan was the maximum concentration that could dissolve in the above acidic medium. The molar concentrations of sialic acid and EDC were based on the theoretical calculation of the number of primary amines in one mole of chitosan calculated from the number of glucosamine units. To achieve different percentage labeling, the ratio of the amount of sialic acid added to primary amines in reaction was varied in each case. For the experiment, sialic acid at different molar concentrations was dissolved in 1ml of activation buffer (0.1M of 2-[morpholino]ethanesulfonic acid (MES) and 0.5M NaCl at pH 6.0). To this activated buffer solution, 0.3626mM of EDC (10 fold excess to the moles of primary amines in chitosan) and 5mM of Sulfo-NHS (1.1mg) were added. The pH was maintained in between 5.0 to 6.0 by using 0.1M phosphate buffer (at pH 7.2). The reaction mixture was continuously rotated at room temperature for the activation of carboxyl

groups and formation of semi-stable amine-reactive NHS ester intermediate. After 15 min, 1.4µl of 2-mercaptoethanol was added to deactivate the unreacted EDC. The reaction mixture was stirred for 2min and then, 1 ml of chitosan solution (at 8 mg/ml) was added; pH raised to 7.0 by the use of phosphate buffer and mixture was allowed to react overnight. After 24h, the reaction mixture was checked for precipitation. If observed, the resulting precipitate was dissolved by drop-wise addition of 10% (v/v) acetic acid solution before purification.

To remove the unreacted sialic acid and EDC, the reaction volume was ultrafiltered using 10000 NMWL cutoff Amicon Ultra Centrifugal filter unit. Six washes of DI water were done each time, assuming that the final concentration of the free sialic acid in the mixture was less than 6% of the total sialic acid (free and covalently bound to chitosan) (Patel, Henry et al. 2006). After purification, the sialic acid labeled chitosan was stored at -4 ^{o}C for later use.

3.1.3. Warren Assay to Determine the Extent of Sialic Acid Labeling

As we have successfully labeled chitosan with sialic acid, it is crucial to determine the amount of sialic acid that is present on chitosan for further studies. The extent of sialic acid labeling was determined using the procedure described by L. Warren (Warren 1959). In this method, the free sialic acid undergoes periodic oxidation resulting in the formation of β-formylpyruvic acid. This acid reacts with 2 molecules of thiobarbituric acid to give a red chromophore with a maximum absorbance at 549nm (Okennedy 1979). The Warren assay measures only free sialic acids and hydrolysis of the sample must be done to liberate the sialic acids from their bound state. Also, Warren assay on hydrolyzed and unhydrolysed samples can be performed to give an estimate of free, bound and total sialic acid in a sample. The best estimate from the Warren assay can be obtained if the concentration of sialic acid in sample is in between 0.05mM and 0.3mM.

The Warren assay uses the following solutions

a) 0.2M Sodium (meta) periodate in 9M phosphoric acid (prepared fresh each time)
b) 10% (w/v) Sodium arsenite in a solution of 0.5M sodium sulphate-0.1M H_2SO_4
c) 0.6% (w/v) Thiobarbituric acid in a solution of 0.5M sodium sulphate

The procedure to determine the extent of sialic acid labeling is as follows (Warren 1959; Okennedy 1979):

1. Assuming that the complex synthesized was 100% labeled, a sample containing 0.3mM of sialic acid concentration was hydrolyzed by 0.1N hydrochloric acid at 80 ^{o}C for 1h.
2. To a 0.2ml hydrolyzed sample, 0.1ml of periodate solution (a) was added. The tubes were shaken and allowed to stand at room temperature for 20min.
3. After that, 1ml of arsenite solution (b) was added and the tubes shaken till the yellow-brown color disappears. Care was taken to completely make the yellow-brown color disappear. This was done by immediately vortexing after adding the arsenite, allowing the solution to stand for a few minutes and again vortexing.
4. Thiobarbituric acid solution (c), 3ml was added, the solution intensely mixed and the tubes immersed in a vigorously boiling water bath for 15min. Samples which showed the presence of white coloration were discarded as it indicated that the yellow-brown color was not completely removed in the earlier step.
5. The tubes were placed in cold water for 5min to develop the chromophore.
6. Cyclohexanone, 4.3ml was added and tubes shaken vigorously.
7. Samples were then centrifuged at 1500g for 7min at 25 ^{o}C, to extract the resulting red chromophore into cyclohexanone.

8. The precipitate-free upper organic phase was taken in a 10mm path-length quartz fluorometer cell (Starna cells Inc.) and the optical density measured at 549nm. Each reading was repeated 4 times.

Using the same procedure, the assay was performed on pure sialic acid samples to get the standard curve and on pure chitosan to determine whether chitosan interfered with the chromophore production. Three or more independent measurements were taken in each case.

3.2. Results and Discussion

One of major goals of this thesis is to confirm the presence of sialic acid on chitosan backbone. Towards this end, the qualitative verification is done by using FTIR and the quantitative verification is done by using Warren assay.

3.2.1. Verification of Sialic Acid Conjugation to Chitosan

The qualitative analysis of the presence of sialic acid on chitosan was performed using Thermo Electron Nicolet 380 FTIR with Smart Orbit attachment (Thermo Electron Corporation, Waltham, MA). For the FTIR analysis, samples of sialic acid-chitosan conjugates (called complex) were lyophilized using Labconco FreeZone 1 Liter Benchtop Freeze Dry Systems. Dry lyophilized complex powders were stored at -4 ^{o}C before and after use in the FTIR. For the FTIR analysis of chitosan, dry powder obtained directly from the manufacturer was used.

The figure 12 shows the FTIR results for complex B (synthesized using moles of sialic acid to moles of primary amines in chitosan = 1) and complex E (synthesized using moles of sialic acid to moles of primary amines in chitosan = 10). The FTIR result for pure chitosan is also included for comparison purposes. The dashed (red) line indicates the sialic acid-chitosan complex spectra whereas the solid (blue) line shows the pure chitosan spectra.

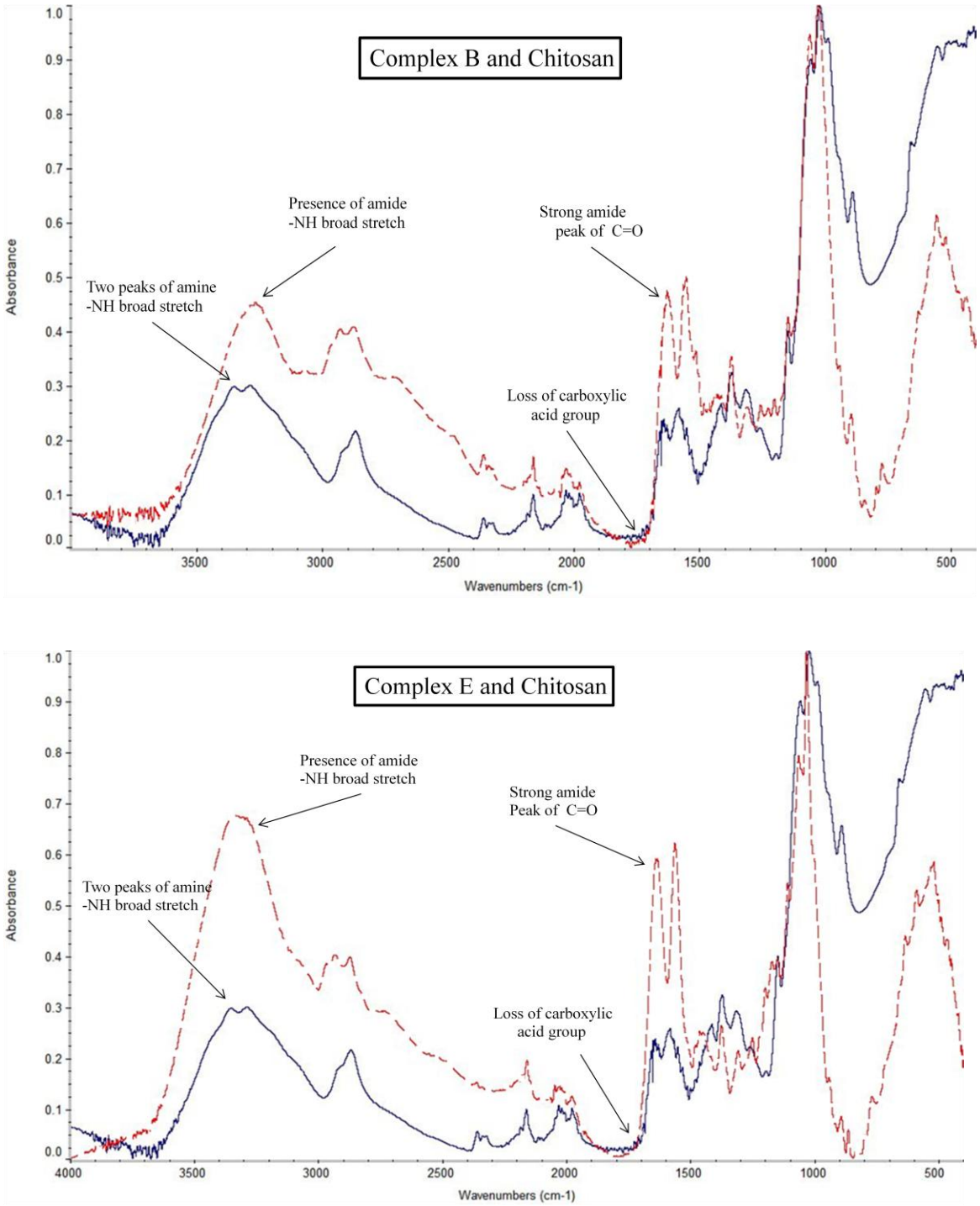

Figure 12: FTIR results of sialic acid conjugated chitosan complex (dashed red line) and pure chitosan (solid blue line).

Complex B (synthesized using moles of sialic acid to moles of primary amines in chitosan = 1)

Complex E (synthesized using moles of sialic acid to moles of primary amines in chitosan = 10)

In the region from 3000 to 3500cm^{-1}, pure chitosan FTIR shows two weak peaks that are indicative of the primary amines present in chitosan. These two peaks are absent in complex spectra and instead a broad band for amide –NH stretching frequency is observed. A strong peak around ~1650cm^{-1} for the complex spectra, indicated the presence of amide bond. The loss of primary amines and the formation of amide bonds confirmed the complex of SA with chitosan. Sialic acid has carboxylic acid group which is used to couple with the amine in chitosan. In the complex spectra, the characteristic strong peak of C=O of carboxylic acid in between 1700 to 1725cm^{-1} is not observed. The complex also shows peaks at ~1030cm^{-1} and ~1380cm^{-1} that indicates the presence of alcohols and acids consistent with sugar molecule attached to chitosan.

The spectra for intermediate concentrations of sialic acid (i.e. 1/4th fold, 2 fold, 4 fold) are not shown (but verified). From these results, we can qualitatively say that the EDC chemistry was successful for the synthesis of sialic acid conjugated chitosan. However, by using FTIR only qualitative results were obtained and it was not possible to distinguish between different samples from their respective spectra. For the quantitative estimation, the Warren assay is used to measure the sialic acid present on chitosan.

3.2.2. Quantification of the Extent of Sialic Acid Conjugation

The Warren assay was performed on pure sialic acid to generate the standard curve. As seen from figure 13, the Warren assay gives very accurate and reproducible results with very low standard deviation. The standard curve was useful in the calculation of the degree of labeling of chitosan. The assay gave accurate results if the concentration of sialic acid in the sample was in between 0.05mM to 0.3mM. The assay was also performed on higher SA concentrations, but the absorbance curve saturated limiting the highest concentration that can be tested to 0.3mM (results not shown). Pure chitosan was also evaluated using the Warren assay but the results were

negative indicating that chitosan did not interfere with the assay. In synthesizing the chitosan-sialic acid complex, we postulated that by varying the ratio of moles of sialic acid to the moles of primary amine in the reaction mixture, we can achieve different degrees of labeling of chitosan with SA.

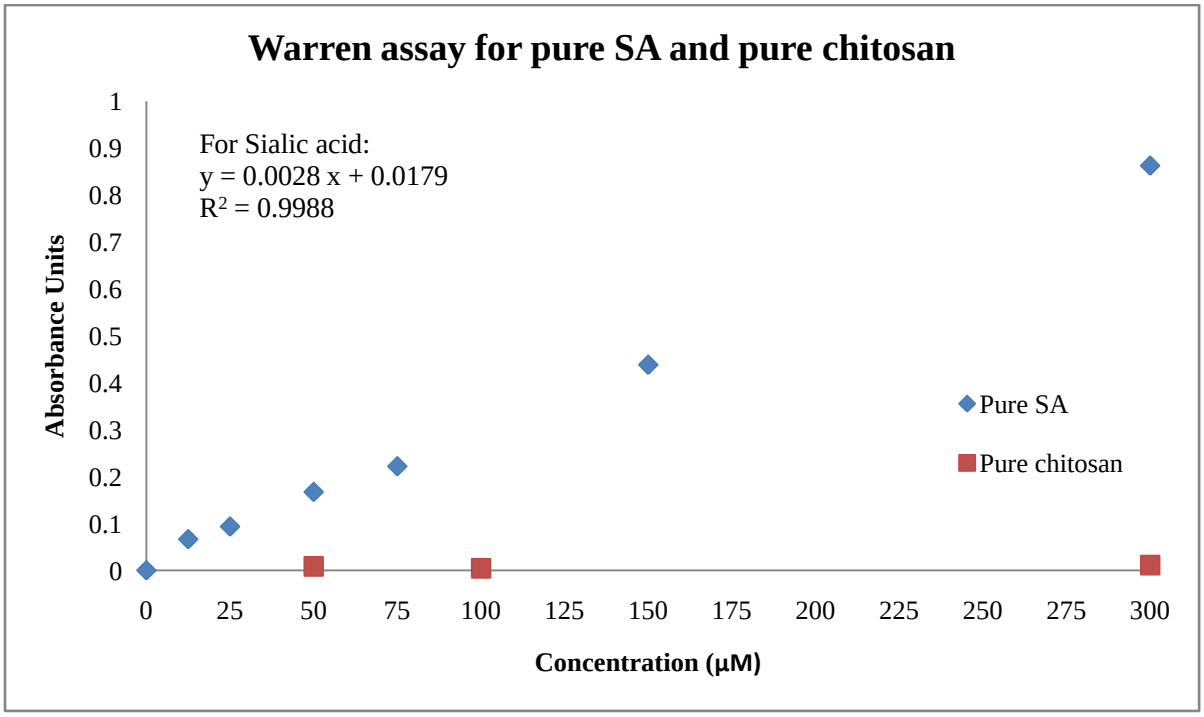

Figure 13: Warren assay for pure sialic acid and pure chitosan

The moles of primary amines in reaction mixture were determined theoretically from the degree of deacetylation of the chitosan sample. This extent of sialic acid (SA) labeling was effectively determined by using the Warren assay as the FTIR results were insufficient for the quantitative estimate of the amount of SA present on chitosan. The percentage of SA labeling was calculated by dividing the absorbance of the sample by the estimated absorbance for 100% labeling of the amine terminals of the chitosan with sialic acid. The percentage of sialic acid conjugation for different samples of complex synthesized by the EDC chemistry is shown in Table 3.

Table 3: Percentage Labeling of Sialic Acid Conjugated Chitosan

Sample ID	Moles of sialic acid added to reaction / Moles of primary amines in chitosan	Percentage Labeling
A.	0.25	7.97
B.	1	14.43
C.	2	38.15
D.	4	41.46
E.	10	49.02

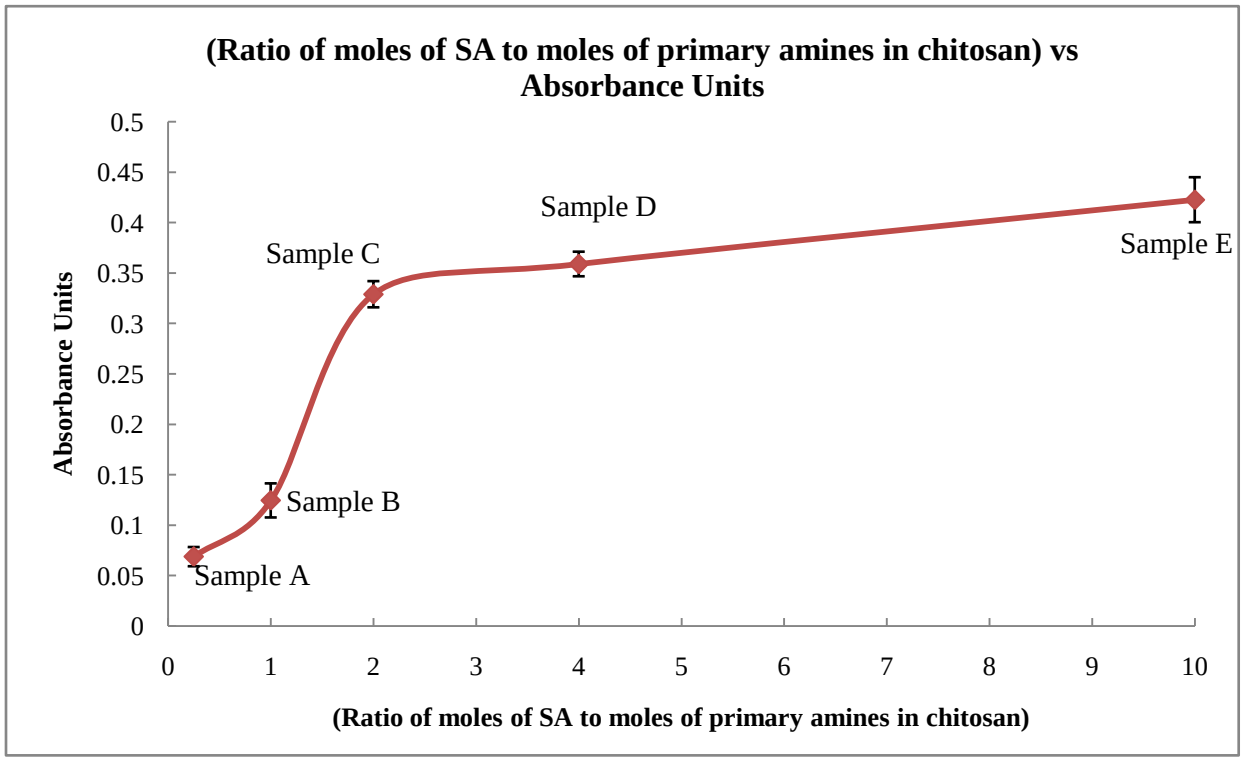

Figure 14: Warren assay for different samples of complex synthesized. Three or more independent measurements were taken.

From the graph in figure 14, we observe that the conjugation chemistry follows a classic saturation curve for sialic acid labeling. As the concentration of sialic acid increases, the degree of labeling increases until the curve starts saturating. The results also indicate that the saturation characteristic appear after sample C (with ratio of moles of SA to moles of primary amines = 2). It may be possible that as more and more sialic acids get attached to chitosan via amine groups, it

becomes increasingly difficult to accommodate additional sialic acids. It seems unlikely that we would achieve a higher degree of labeling of chitosan by SA as the bulk of SA's already present on the chitosan would prevent free SA from interacting with the amines on chitosan due to steric hindrance. This direct proportionality of SA labeling is beneficial such that it will allow us to study the effect of SA clustering on Aβ toxicity. Studies have shown that the binding affinities of Aβ for sialic acids in gangliosides increased in clustered or multivalent regions of the membrane compared to membrane regions where sialic acids or gangliosides were unclustered (Ariga, Kobayashi et al. 2001; Kakio, Nishimoto et al. 2001; Kakio, Yano et al. 2004; Ariga, McDonald et al. 2008). Thus, by using complexes of varying degrees of sialylation, the properties of Aβ affinity could be thoroughly investigated. This was not possible for dendrimer labeled SA in the work of Patel et al (Patel, Henry et al. 2006). Also, it will be possible to predict the concentration of SA, the degree of surface modification required that is optimum for attenuating Aβ toxicity.

Finally, it is proposed that even though chitosan has been modified, it retains its original properties such as biocompatibility and non-toxicity. This is reasonable argument as the basic skeleton structure of chitosan remains unchanged. By attaching sialic acids, we postulate that the construct will have better and improved properties which can be useful in attenuating Aβ toxicity. The additional advantage of using chitosan would be that we can isolate and study the effects of just sialic acids as the chitosan backbone remains the same in each case. The testing of this complex in-vitro with and without Aβ addition is discussed in the next sections.

4. INITIAL STUDIES ON SH-SY5Y NEUROBLASTOMA CULTURES

4.1. Experimental Procedures

4.1.1. Materials

Aβ(1-40).HCl peptide was purchased from Anaspec Inc. (San Jose, CA). Human neuroblastoma SH-SY5Y cells were purchased from ATCC (Manassas, VA). Cell dissociation buffer and cell culture reagents were purchased from Gibco-Invitrogen (Grand Island, NY). Human recombinant nerve growth factor-β (NGF-β), (3-[4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and all other chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO).

4.1.2. Peptide Preparation

The Aβ peptides were prepared analogously to established methods in structural and toxicity literature for forming β-sheet and Aβ fibrils. Aβ(1-40) stock solutions were prepared by dissolving the lyophilized peptide in anhydrous dimethyl sulfoxide (DMSO) to make 10mg/ml stock solutions. After incubating for 30 min to 1 hour at 25 ^{o}C, stock solutions of Aβ were diluted directly to their final concentrations in sterile cell culture medium and rotated at 25 ^{o}C for 24h prior to addition to the cells. This method of peptide preparation yielded Aβ fibrils and other aggregated species that were consistently toxic to the cells in culture at concentrations between 20 and 100μM (Rymer and Good 2001; Patel, Henry et al. 2006).

4.1.3. Cell Culture

Human neuroblastoma SH-SY5Y cells were cultured in a humidified 5% CO_2/air incubator at 37 ^{o}C in Minimum Essential Media (MEM), supplemented with 10% (vol/vol) fetal bovine serum, 2.2 mg/ml $NaHCO_3$, 100 μl/ml penicillin, 100 μg/ml streptomycin and 2.5 μg/ml amphotericin-B (fungizone). SH-SY5Y cells were NGF differentiated prior to use in toxicity

experiments by addition of 20 ng/ml NGF to cells for 5-7 days in 96 well plates. All the cells used in experiments were below passage 10.

4.1.4. Procedure for Optimization of MTT Toxicity Assay

The (3-[4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay is widely used in toxicity studies. Though it is a very established and proven viability assessing assay, the protocols and methods used vary from place to place, cell type, and the nature of the experiment etc. The MTT assay allows the user to get estimates of viable cell numbers by using standard microplate absorbance readers. Viable cells metabolically reduce the yellow MTT salt giving purple/blue colored crystals which are solubilized by using appropriate solvents and reagents such as Triton X-100, DMSO, isopropanol etc. to give a colored product. The resulting colored solution absorbance is correlated to the number of viable cells in the sample (Mosmann 1983; Liu, Peterson et al. 1997; Datki, Juhasz et al. 2003). The MTT assay is used to determine the viability of SH-SY5Y cells in toxicity experiments. To determine the optimum cell number and incubation time to use for the SH-SY5Y cells the following procedure was used.

1. Undifferentiated SH-SY5Y cells were cultured to about 80% confluency by using culturing method described in above sections.
2. A stock solution of $5x10^6$ cells/ml was prepared in cell culture medium.
3. Using the stock solution prepared, cells were seeded in two 96 well plates at densities ranging from $50x10^3$ to $2x10^3$ cells/well using 100µl of cell culture medium. 4 independent reading were taken and control wells of cell culture medium were included.
4. The plates were returned to the incubator to give time for the cells to reattach.
5. After 24h, the cell culture medium was aspirated and replaced by 100µl of cell culture medium without phenol red.

6. MTT was dissolved at a concentration of 5mg/ml in cell culture medium w/o phenol red.
7. 10µl of this freshly prepared MTT reagent was added to each well.
8. One plate was incubated for 1.5h and other for 4h at 37 ^{o}C.
9. After incubation, the cell culture media was replaced with 200µl of DMSO to dissolve purple crystals formed.
10. After 15 min on the shaker, absorbance was measured on a standard micoplate reader at 570nm and background absorbance at 690nm.
11. Substract background, determine the average of 4 readings and substract the average value for the blank.
12. Plot Absorbance against cell number/well.

4.1.5. Initial Studies in Attenuating Aβ Toxicity by Complex E

SH-SY5Y cells were plated at a density of $2x10^4$ cells/well in 96 well plates and NGF differentiated. After 5-7 days differentiation, culture medium was replaced with medium containing NGF to which the compound to be tested was added, either Aβ, chitosan, sialic acid conjugated chitosan complex, or a combination of the above. Aβ peptide at a concentration of 50µM was prepared by methods described in earlier sections. In all experiments, Aβ was added to the cells approximately 30min prior to the addition of chitosan or conjugated complex. A gradient of chitosan and conjugated complex from 100 to 2µM was applied on the 96 well plate. After 24h, of the addition of Aβ, chitosan, sialic acid conjugated chitosan, the viability of cells was determined by using the MTT assay. The media from the wells was replaced with 100µl of culture media without phenol red. 10µl of 5mg/ml MTT solution was freshly prepared in culture media without phenol red and added to all the wells. After incubation for 2h, the cells were checked for purple crystals and the media was replaced with 200µl of DMSO. After 20min on

the shaker, absorbance at 570nm and 690nm was measured using a standard microplate reader. Normalized viability values were obtained by dividing the percentage of viable cells in the sample by that in the control samples with no Aβ or other agent added.

4.2. Results and Discussion

4.2.1. Optimization of MTT Assay for Cell Number and Incubation Time

From figure 15, we see that the linear range for the MTT assay of SH-SY5Y cells is in between $5x10^3$ to $20x10^3$ cells/well. Results for higher concentration of cell numbers show uneven proportionality and higher variance in absorbance readings. At $20x10^3$ cells/well, one observes good agreement in the graphs of different incubation times. Also, it is impractical in using less than $20x10^3$ cells/well as the number would be insufficient for toxicity studies. Hence, we decided to use $2x10^4$ cells/well for future studies.

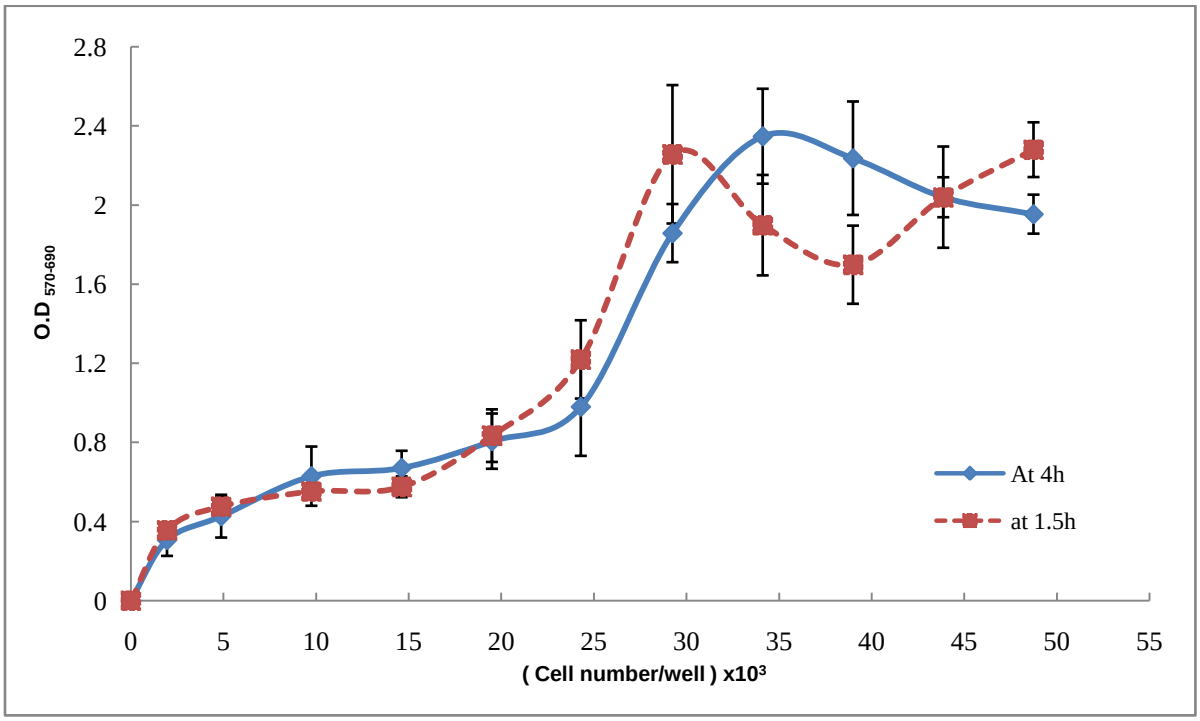

Figure 15: MTT assay optimization for determining optimum cell number per well and optimum incubation time for SH-SY5Y cells. 4 independent measurements were taken.

Considering the linear range for SH-SY5Y cells, increase of incubation time from 1.5h to 4h does not show any marked improvement in absorbance values. As a result, the incubation time for toxicity studies was chosen to be 2h.

4.2.2. Toxicity of Chitosan and Sialic Acid Conjugated Chitosan

One of the goals of this research was to develop a compound that was not only able to inhibit Aβ toxicity but was biocompatible as well. Considering this, the toxicity of chitosan with and without sialic acid modifications was assessed using differentiated SH-SY5Y cells before studying its Aβ toxicity attenuation properties. Though complexes were synthesized with different degrees of labeling, only complex E (Ratio of moles of sialic acid to moles of primary amines =10) was used for initial studies. Representative data for chitosan and complex E are shown in figure 16.

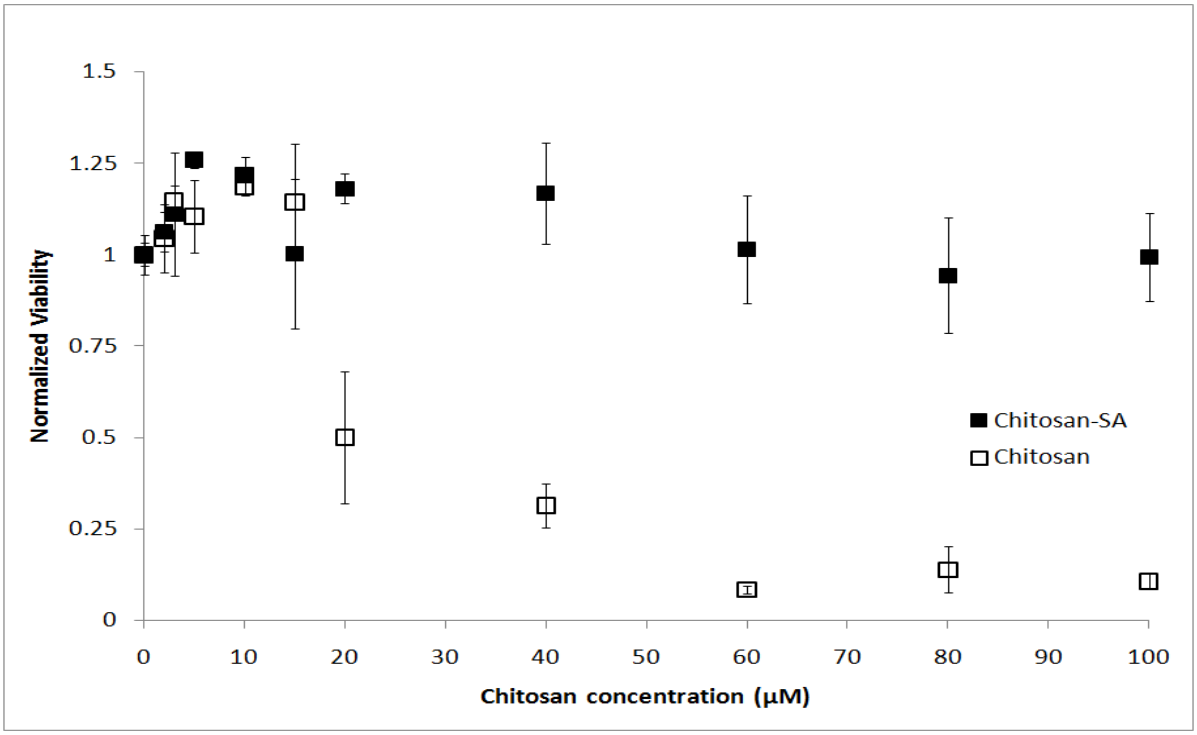

Figure 16: Normalized viability of differentiated SH-SY5Y cells treated with sialic acid conjugated chitosan i.e. sample E (■) and unmodified chitosan (□)

From the results, we observe that pure chitosan shows minimal toxicity below 15μM whereas the viability starts decreasing as concentration increases. This loss of viability can be explained by the fact that chitosan being a polycation could be interacting strongly with the negatively charged cell membrane leading to loss of viability. Then, this loss of viability at increasing concentration would be related to the number of terminal amine groups on chitosan molecule that are interacting with the cells. It is posited that polycation toxicity is a strong function of both mass and cationic charge density (Fischer, Li et al. 2003). On the other hand, complex E (compound synthesized using 10 fold excess sialic acid to primary amines) showed almost no loss of viability. From the Warren assay, it was determined that complex E has nearly 49% sialic acid labeling of the chitosan amine terminals. The labeling of sialic acids would balance the polycation strength and this must have contributed to its significant improvement in viability over unlabeled chitosan. In further experiments, it will be beneficial to study the toxicity properties of different sialic acid labeled complexes as this data can further help in understanding how modification of polycation strength can influence toxicity.

4.2.3. Attenuating the Toxicity of Aβ by Sialic Acid Conjugated Chitosan

We hypothesized that multivalent sialic acid polymers can mimic the cell membrane, and would effectively compete for Aβ binding. A number of studies have shown that Aβ interacts with cell membranes through gangliosides or glycoproteins containing sialic acids (Yanagisawa, Odaka et al. 1995; Choo-Smith, Garzon-Rodriguez et al. 1997; Choo-Smith and Surewicz 1997; Ariga, Kobayashi et al. 2001; Wakabayashi, Okada et al. 2005; Ariga, McDonald et al. 2008). Also, the binding affinity of Aβ to membranes was higher when multiple sialic acids were present, either because of clustering of gangliosides or because of the degree of sialylation of the gangliosides (Ariga, Kobayashi et al. 2001; Kakio, Nishimoto et al. 2001; Kakio, Yano et al.

2004). Thus, the goal is to synthesize a membrane mimicking compound, which would interact with Aβ in a more favorable manner than cell surface thereby protecting cells from toxic effects of Aβ. To that end, the ability of chitosan and sialic acid conjugated chitosan to attenuate toxicity of 50μM aggregated Aβ was assessed when added to differentiated SH-SY5Y cells. The Aβ toxicity attenuation at different concentrations of chitosan and complex E is shown in figure 17. It is observed that the protective effect for complex E increases up to around 20μM and then starts decreasing. Also, complex E offers better toxicity attenuation than chitosan alone at lower concentrations. The toxicity attenuation property of the complex is attributed to the increased sialic acid valency on chitosan.

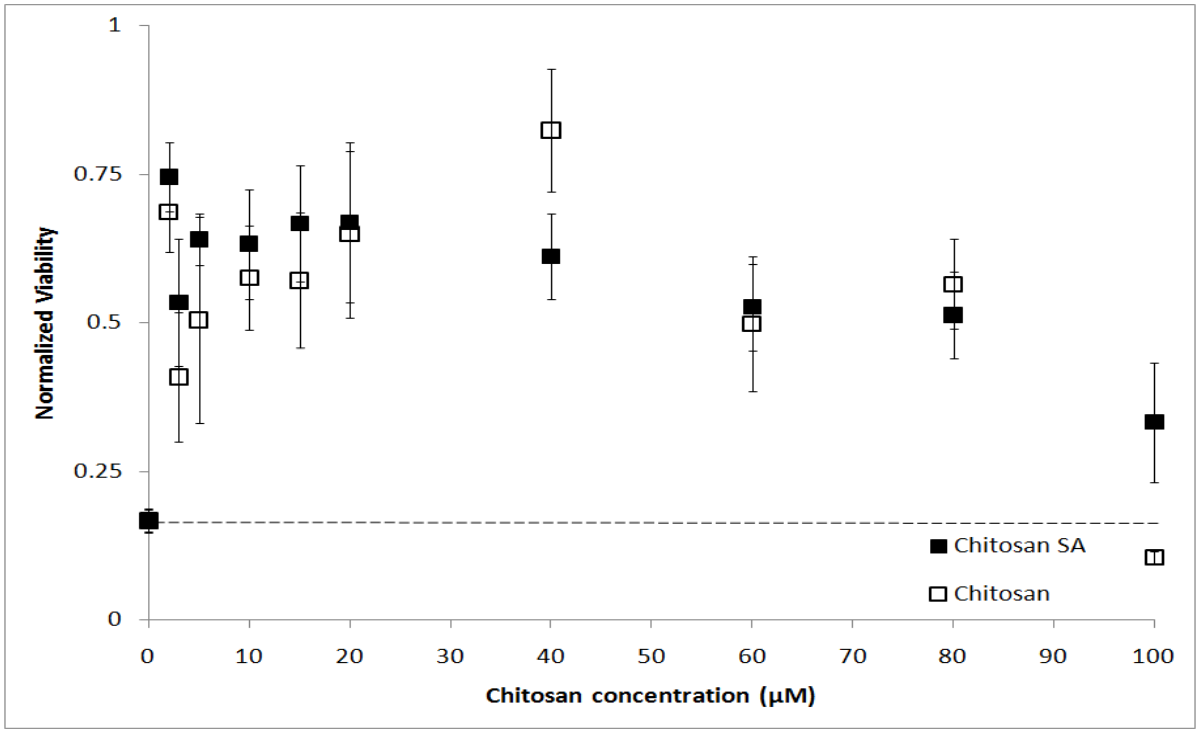

Figure 17: Normalized cell viability of differentiated SH-SY5Y cells upon treatment with 50μM Aβ with sialic acid conjugated chitosan i.e. complex E (■) and unmodified chitosan (□). Dashed line represents the viability of cells treated with Aβ alone.

There can be several possibilities that can explain the protective effect shown by the complex. It is possible that the complex binds to Aβ via a competitive mechanism that

effectively reduces the concentration free Aβ that binds with the cell. Another mechanism would be that Aβ binds to the mimic, which then interact with the cells, but it is less toxic than Aβ interacting directly with the cells. Another possibility is that as the mimic contains multiple charges, it could act as an effective shield for the electrostatic interactions between Aβ and the cell. From our initial results, it is not possible to distinguish between the exact mechanism or the combined mechanisms that are attenuating toxicity. Further studies with different complexes at various Aβ concentrations would help in elucidating the mechanisms active in attenuating toxicity.

The protective effect of chitosan is not surprising. It has been suggested that cell surface binding of Aβ is dictated by electrostatic interactions, and preventing these interactions decreases Aβ toxicity (Hertel, Terzi et al. 1997; Patel, Henry et al. 2006). Chitosan being a polycation can interact with both Aβ and cell membrane via electrostatic interactions. The positively charged chitosan backbone could interact with the negatively charged cell membrane, shielding the cell from Aβ binding and, therefore, blocking toxicity. However, it can also be possible that this interaction with cell surface can lead to cell death, but at a lower rate than Aβ. Looking at figures 16 and 17, we can observe that at concentrations above 20μM, cells treated chitosan alone have lower viability than cells treated with both Aβ and chitosan. It is possible that when cells are treated with both Aβ and chitosan, the polycation interacts with both Aβ and cell surface, both of which are negatively charged. Thus, in this case, the interaction of chitosan with Aβ minimizes cell-Aβ interactions and also causes reduction in polycation strength of chitosan that interacts with the negatively charged cell membrane leading to higher viabilities. In case of just treatment with chitosan, the strong polycation interacts with only the cell membrane and thus has more toxicity. This can explain why higher viability was observed in the case of Aβ, chitosan treatment of cells as compared to treatment with just chitosan. Also, this study was performed on

50μM Aβ concentration. Looking further, for in-vivo treatment, the concentration of Aβ would be several times lower than the concentration used in our studies. Then, in such a case, treatment with only chitosan would be more toxic than the in-vivo Aβ concentration towards cells. In such cases, sialic acid conjugated chitosan would be a far better choice as it displays not only protective properties but also biocompatibility. Hence, the complex synthesized would prove as a better choice for further tests.

Thus, regardless of the mechanism by which sialic acid conjugated chitosan attenuates Aβ toxicity, the initial results on complex E suggest that sialic acid conjugated chitosan can effectively attenuate Aβ toxicity without the associated toxicity of chitosan as a backbone. A number of studies have stressed the involvement of multivalent sialic acids containing gangliosides and compounds in attenuating Aβ toxicity. Thus, we have succeeded in synthesizing complexes having different degrees of labeling. The effect of multivalency on toxicity inhibition can be effectively studied using these multivalent complexes synthesized.

From the initial studies, it is unclear whether monomeric, oligomeric or more aggregated (fibril or protofibril) forms of Aβ bind to sialic acid conjugated chitosan. In toxicity experiments, Aβ was prepared in such a way that there was always a mixture of both small and large Aβ oligomers. It is also unknown what form of Aβ binds to gangliosides or GM1 rich regions of cell membranes. It is difficult to prove whether Aβ binds to the mimic at the same site or different site. If it binds at a different site, then Aβ can also interact with the cell contributing to toxicity. If all these questions were properly addressed, it would be possible to synthesize compounds having the same multivalency or the same orientation of sialic acids that is optimum for Aβ toxicity inhibition.

5. CONCLUSIONS

The world's most prominent neurodegenerative disease, Alzheimer's disease, has been the topic of intense research for over a century now. Unfortunately still, its mechanisms and causes are not fully understood and its cure unknown. The work presented in this thesis is a step towards understanding the missing pieces. Much of the work in this field has focused on Aβ peptide, which is posited to play a central role in AD. Crucially, the mechanism by which Aβ causes neurotoxicity is the subject of much debate. One theory is that Aβ toxicity is linked to the formation of toxic species, others believe that Aβ acts via association with the cell membrane causing toxicity. There are also several theorized environmental conditions that lead to the development of AD. However, it is generally agreed upon that, the first step in any of the mechanisms of Aβ action on the cell is Aβ binding to the cell membrane. The understanding is that, Aβ has to interact with the cell to cause neurotoxicity, and this interaction occurs through the cell membrane in a still unexplained manner. It is precisely this theoretical bottleneck region that we plan to target.

The aim of this work is to develop a biomimetic compound having antibody-like affinity towards Aβ that can effectively attenuate the toxicity of Aβ peptide in-vitro, but with smaller molecular size that may be more appropriate for the demands of a neurotherapeutic. To this end, most of this thesis focuses on the development and synthesis of such a biomimetic compound. Using the methods of synthetic chemists, a membrane mimicking compound was synthesized that was multivalent in sialic acids and non-toxic. Preliminary results were obtained on the Aβ toxicity attenuation properties of this compound.

Earlier works demonstrated the use of dendrimers and sialic acid labeled dendrimers in attenuating toxicity. As the dendrimer structure was rigid, it would be possible that the labeling

with sialic acids was suboptimum for Aβ binding. The different dendrimers tested had different levels of intrinsic toxicity towards cells, which affected the toxicity attenuation properties of these constructs. As an improvement over the previous work, chitosan was selected as a backbone as it has demonstrated excellent biocompatible and non-toxic properties. Also, chitosan can be easily modified and the linear polysaccharide chain is relatively flexible.

5.1. Synthesis of Sialic Acid Labeled Chitosan

Chitosan is a versatile polysaccharide that has a unique set of biochemical and physiochemical properties that has allowed its use in a wide variety of applications. Chitosan has amine functional group that can be modified without affecting the fundamental skeleton, thereby retaining its original properties and also acting as a carrier for sialic acid, which is crucial in our work as a possible membrane mimic. Considering this, the amine groups in chitosan were coupled with the carboxyl group in sialic acid to form a stable amide linkage that resulted in to sialic acid conjugated chitosan. The reaction was carried out by the use of EDC, a zero-length crosslinker in buffer solutions whereas sulfo-NHS was used to give a stable intermediate in the coupling process. By varying the ratio of the moles of sialic acid in reaction mixture to the moles of primary amines in chitosan, we succeeded in synthesizing sialic acid labeled chitosan complexes having degree of labeling ranging from 8% to 49% (Table 3). It was found that the conjugation chemistry followed a classic saturation curve for SA labeling (Figure 14). It is unlikely that a higher degree of labeling could be achieved as we postulate that the chitosan surface would be saturated with the already attached sialic acids preventing free sialic acids from attaching to the remaining amines of chitosan. The presence of sialic acids was confirmed by FTIR results which showed the loss of primary amines and presence of amide linkage in complex spectra as compared to the spectra for pure chitosan. The degree of labeling was

verified by the warren assay, which gives a red chromophore whose absorbance can be correlated to the amount of sialic acid present in the sample.

5.2. Initial Results for Aβ Toxicity Attenuation

Chitosan and sialic acid conjugated chitosan complex were added to differentiated SH-SY5Y cells to study their toxic effects. For the initial results, only complex E was used. In the case of pure chitosan, we observed significant loss of viability after 15μM which can be attributed to its strong polycation characteristics. This loss of viability at higher concentrations could be due to the interaction of positively charged chitosan with the negatively charged cell membrane. On the other hand, complex E, which had around 49% labeling of amines by sialic acids, showed minimal loss in viability at all concentrations tested. We believe that labeling with negatively charged sialic acids must have counterbalanced the strong polycation characteristic of chitosan. As a result, we do not see the toxicity characteristics seen for chitosan even at higher concentrations of the complex. Thus, initial results suggest that we were successful in synthesizing a biocompatible cell membrane mimicking compound (Figure 16).

We postulated that sialic acid conjugated chitosan can effectively mimic the cell membrane, thus, binding to Aβ and sequestering it, making Aβ unavailable for the cellular interactions. Using differentiated SH-SY5Y cells, the ability of chitosan and complex E to attenuate the toxicity of 50μM aggregated Aβ was investigated. The complex offered better protection against Aβ as compared to chitosan at lower concentrations. The toxicity attenuating properties of the complex were attributed to the presence of sialic acids on chitosan. As the chitosan complex is a straight chain sialic acid labeled polysaccharide, it would be possible for the complex to adopt conformation that is optimum for Aβ binding. This flexibility offered by chitosan was not observed in earlier works. The protective effect of chitosan could be due to the

blocking of the electrostatic interactions between Aβ and the cell membrane. Though we see a protective effect from both chitosan and sialic acid conjugated chitosan, we have a reason to believe that the mechanisms in attenuating toxicity are different in each case. But, from the initial results it was not possible to identify the exact mechanism involved in attenuating Aβ toxicity. The initial results are in good agreement with those obtained for sialic acid conjugated dendrimers (Patel, Henry et al. 2006) and photocrosslinked oligomers having sialic acids (Cowan, Coté et al. 2008). Also, considering the possibility of in-vivo treatment, we postulate that the complex synthesized could be more effective and biocompatible than unmodified chitosan. These results again stress the importance of surface labeling by sialic acids in attenuating toxicity. We conclude that by the use of chitosan as a polyaminated backbone, we have observed similar (if not better) results with decreased backbone toxicity. These finding encourage further investigation into the development of sialic acid modified and other polysaccharide materials for use in prevention of Aβ toxicity.

6. FUTURE WORK

As with any work esp. research, time is a major limitation, and there are several things that are left to be done. For the work presented in this thesis, I believe we have some short term goals and some long term goals that would need through investigation.

It seems that that one of the best strategies in preventing AD pathogenicity is to eliminate the presence of excess toxic Aβ from the vicinity of neurons. As immunization has proved complicated, it would be fruitful to develop non-immunogenic Aβ sequestering agents having antibody-like affinity. Considering that, in this thesis we examined the feasibility of developing a multivalent sialic acid conjugated polymer that was biocompatible and also able to attenuate Aβ toxicity. Considering the favorable results obtained some of the short term goals are as follows:

- Aβ toxicity attenuating properties of other complexes with different degrees of labeling should be studied. These results can elucidate the relationship between multivalency, optimum sialic acid labeling and toxicity attenuation of Aβ peptide.
- Based on previous results, the efficiencies of different complexes to attenuate toxicity should be investigated. This can be done by fitting the data to a suitable model to give the toxicity inhibition parameters.
- The binding of Aβ to the complex synthesized should be investigated by the use of radiochemical techniques. One such technique, the Bolton Hunter method has been described for Aβ. This would help in the estimation of the equilibrium dissociation constants for our complexes which can be compared with other works.

There are some unanswered questions still left that are deemed beyond the scope of this thesis. Some of these are presented with the possible method of approach. Other long term aims are also given below:

- The effect of various polysaccharide backbones attached with different sugars should be investigated for better toxicity attenuation properties. This can address how differences in sugar structures can alter the binding affinity and mechanism of Aβ binding and, consequently, differences in toxicity.
- Identification of the most toxic form of Aβ and the species that preferably binds with GM1 gangliosides will greatly aid the development of biomimetic compounds such that these compounds have the orientation and attachment optimum for Aβ binding.
- Whether Aβ-conjugate complex can act as a possible seed for aggregation and accumulation of senile plaques should be investigated.
- The question of whether these constructs could be used in-vivo remains to be addressed. The delivery of such multivalent sialic acid polymers across the blood brain barrier (BBB) becomes increasingly difficult as the size of the polymers increase. The option of peripheral treatment of AD, wherein these constructs act as a sink for Aβ in the blood should be investigated as an alternative to overcome the BBB.

REFERENCES

Alphonse Probst, D. L., Jürg Ulrich, (1991). "Alzheimer's Disease: A Description of the Structural Lesions." Brain Pathology **1**(4): 229-239.

Antzutkin, O. N., R. D. Leapman, et al. (2002). "Supramolecular Structural Constraints on Alzheimer's beta-Amyloid Fibrils from Electron Microscopy and Solid-State Nuclear Magnetic Resonance." Biochemistry **41**(51): 15436-15450.

Ariga, T., K. Kobayashi, et al. (2001). "Characterization of high-affinity binding between gangliosides and amyloid beta-protein." Archives of Biochemistry and Biophysics **388**(2): 225-230.

Ariga, T., M. P. McDonald, et al. (2008). "Thematic Review Series: Sphingolipids. Role of ganglioside metabolism in the pathogenesis of Alzheimer's disease--a review." J. Lipid Res. **49**(6): 1157-1175.

Bard, F., C. Cannon, et al. (2000). "Peripherally administered antibodies against amyloid [beta]-peptide enter the central nervous system and reduce pathology in a mouse model of Alzheimer disease." Nat Med **6**(8): 916-919.

Barten, D. and C. Albright (2008). "Therapeutic Strategies for Alzheimer's Disease." Molecular Neurobiology **37**(2): 171-186.

Bateman, D., J. McLaurin, et al. (2007). "Requirement of aggregation propensity of Alzheimer amyloid peptides for neuronal cell surface binding." BMC Neuroscience **8**(1): 29.

Berchtold, N. C. and C. W. Cotman (1998). "Evolution in the Conceptualization of Dementia and Alzheimer's Disease: Greco-Roman Period to the 1960s." Neurobiology of Aging **19**(3): 173-189.

Blennow, K., M. J. de Leon, et al. (2006). "Alzheimer's disease." The Lancet **368**(9533): 387-403.

Boss, M. A. (2000). "Diagnostic approaches to Alzheimer's disease." Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease **1502**(1): 188-200.

Braak, F., H. Braak, et al. (1994). "A sequence of cytoskeleton changes related to the formation of neurofibrillary tangles and neuropil threads." Acta Neuropathologica **87**(6): 554-567.

Brody, D. L. and D. M. Holtzman (2008). "Active and Passive Immunotherapy for Neurodegenerative Disorders." Annual Review of Neuroscience **31**(1): 175-193.

Chi, E. Y., S. L. Frey, et al. (2007). "Ganglioside GM1-Mediated Amyloid-beta Fibrillogenesis and Membrane Disruption." Biochemistry **46**(7): 1913-1924.

Choo-Smith, L.-P. i., W. Garzon-Rodriguez, et al. (1997). "Acceleration of Amyloid Fibril Formation by Specific Binding of Abeta -(1-40) Peptide to Ganglioside-containing Membrane Vesicles." J. Biol. Chem. **272**(37): 22987-22990.

Choo-Smith, L.-P. i. and W. K. Surewicz (1997). "The interaction between Alzheimer amyloid [beta](1-40) peptide and ganglioside GM1-containing membranes." FEBS Letters **402**(2-3): 95-98.

Cole, S. L. and R. Vassar (2008). "BACE1 Structure and Function in Health and Alzheimer's Disease." Current Alzheimer Research **5**(2): 100-120.

Coles, M., W. Bicknell, et al. (1998). "Solution Structure of Amyloid Beta Peptide (1-40) in a Water-Micelle Environment. Is the Membrane-Spanning Domain Where We Think It Is?" Biochemistry **37**(31): 11064-11077.

Cowan, C. B., G. L. Coté, et al. (2008). "Development of photocrosslinked sialic acid containing polymers for use in A[beta] toxicity attenuation." Biomaterials **29**(24-25): 3408-3414.

Cowan, C. B., D. A. Patel, et al. (2009). "Exploring the mechanism of beta-amyloid toxicity attenuation by multivalent sialic acid polymers through the use of mathematical models." Journal of Theoretical Biology **258**(2): 189-197.

Craig-Schapiro, R., A. M. Fagan, et al. "Biomarkers of Alzheimer's disease." Neurobiology of Disease **In Press, Corrected Proof**.

Crino, P. B., M. D. Ullman, et al. (1989). "Brain Gangliosides in Dementia of the Alzheimer Type." Archives of Neurology **46**(4): 398-401.

Cummings, J. (2004). "Alzheimer's Disease." The New England Journal of Medicine **351**(1): 56-67.

Dahm, R. (2006). Alois Alzheimer and the beginnings of research into Alzheimer's disease. Alzheimer: 100 years and beyond. M. Jucker, K. Beyreuther, C. Haass, R. M. Nitsch and Y. Christen, Springer**:** 37-49.

Datki, Z., A. Juhasz, et al. (2003). "Method for measuring neurotoxicity of aggregating polypeptides with the MTT assay on differentiated neuroblastoma cells." Brain Research Bulletin **62**(3): 223-229.

Du Yan, S., J. Fu, et al. (1997). "An intracellular protein that binds amyloid-[beta] peptide and mediates neurotoxicity in Alzheimer's disease." Nature **389**(6652): 689-695.

Eckert, G. P., W. E. Muller, et al. (2007). "Cholesterol-lowering drugs and Alzheimer's disease." Future Lipidology **2**(4): 423-432.

Findeis, M. A. (2007). "The role of amyloid [beta] peptide 42 in Alzheimer's disease." Pharmacology & Therapeutics **116**(2): 266-286.

Fischer, D., Y. X. Li, et al. (2003). "In vitro cytotoxicity testing of polycations: influence of polymer structure on cell viability and hemolysis." Biomaterials **24**(7): 1121-1131.

Ghanta, J., C.-L. Shen, et al. (1996). "A Strategy for Designing Inhibitors of beta -Amyloid Toxicity." J. Biol. Chem. **271**(47): 29525-29528.

Gilman, S., M. Koller, et al. (2005). "Clinical effects of A{beta} immunization (AN1792) in patients with AD in an interrupted trial." Neurology **64**(9): 1553-1562.

Glenner, G. G. and C. W. Wong (1984). "Alzheimer's disease: Initial report of the purification and characterization of a novel cerebrovascular amyloid protein." Biochemical and Biophysical Research Communications **120**(3): 885-890.

Goedert, M. and M. G. Spillantini (2006). "A Century of Alzheimer's Disease." Science **314**(5800): 777-781.

Grabarek, Z. and J. Gergely (1990). "Zero-length crosslinking procedure with the use of active esters." Analytical Biochemistry **185**(1): 131-135.

Grundke-Iqbal, I., K. Iqbal, et al. (1986). "Abnormal phosphorylation of the microtubule-associated protein tau (tau) in Alzheimer cytoskeletal pathology." Proceedings of the National Academy of Sciences of the United States of America **83**(13): 4913-4917.

Grundman, M. and P. Delaney (2002). "Antioxidant strategies for Alzheimer's disease." Proceedings of the Nutrition Society **61**(02): 191-202.

Hardy, J. and D. J. Selkoe (2002). "The Amyloid Hypothesis of Alzheimer's Disease: Progress and Problems on the Road to Therapeutics." Sci. Aging Knowl. Environ. **2002**(29): or8-.

Hardy, J. A. and G. A. Higgins (1992). "Alzheimer's disease: The amyloid cascade hypothesis." Science **256**(5054): 184.

Harvey, R. J., M. Skelton-Robinson, et al. (2003). "The prevalence and causes of dementia in people under the age of 65 years." J Neurol Neurosurg Psychiatry **74**(9): 1206-1209.

Hawkes, C. A., V. Ng, et al. (2009). "Small Molecule Inhibitors of A beta-Aggregation and Neurotoxicity." Drug Development Research **70**(2): 111-124.

Hermanson, G. T. (2008). Zero-Length Crosslinkers. Bioconjugate Techniques (Second Edition). New York, Academic Press**:** 213-233.

Hertel, C., E. Terzi, et al. (1997). "Inhibition of the electrostatic interaction between beta-amyloid peptide and membranes prevents beta-amyloid-induced toxicity." Proceedings of the National Academy of Sciences of the United States of America **94**(17): 9412-9416.

Honig LS, M. R. (2001). "Natural History of Alzheimer's disease." Aging (Milano) **13**: 171-182.

Huang, T. H. J., D.-S. Yang, et al. (2000). "Alternate Aggregation Pathways of the Alzheimer beta -Amyloid Peptide. An In Vito Model of Preamyloid." J. Biol. Chem. **275**(46): 36436-36440.

Iqbal, K., A. del C. Alonso, et al. (2005). "Tau pathology in Alzheimer disease and other tauopathies." Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease **1739**(2-3): 198-210.

Irie, K., K. Murakami, et al. (2005). "Structure of beta-amyloid fibrils and its relevance to their neurotoxicity: Implications for the pathogenesis of Alzheimer's disease." Journal of Bioscience and Bioengineering **99**(5): 437-447.

Ishiguro, K., A. Omori, et al. (1991). "A serine/threonine proline kinase activity is included in the tau protein kinase fraction forming a paired helical filament epitope." Neuroscience Letters **128**(2): 195-198.

Jellinger, K. A. (2004). "Traumatic brain injury as a risk factor for Alzheimer's disease." J Neurol Neurosurg Psychiatry **75**(3): 511-a-512.

K. Iqbal, I. G.-I. (2008). "Alzheimer neurofibrillary degeneration: significance, etiopathogenesis, therapeutics and prevention." Journal of Cellular and Molecular Medicine **12**(1): 38-55.

Kakio, A., S.-i. Nishimoto, et al. (2001). "Cholesterol-dependent Formation of GM1 Ganglioside-bound Amyloid beta -Protein, an Endogenous Seed for Alzheimer Amyloid." J. Biol. Chem. **276**(27): 24985-24990.

Kakio, A., Y. Yano, et al. (2004). "Interaction between amyloid beta-protein aggregates and membranes." Journal of Peptide Science **10**(10): 612-621.

Kayed, R., E. Head, et al. (2003). "Common Structure of Soluble Amyloid Oligomers Implies Common Mechanism of Pathogenesis." Science **300**(5618): 486-489.

Kim, I. Y., S. J. Seo, et al. (2008). "Chitosan and its derivatives for tissue engineering applications." Biotechnology Advances **26**(1): 1-21.

Klafki, H.-W., M. Staufenbiel, et al. (2006). "Therapeutic approaches to Alzheimer's disease." Brain **129**(11): 2840-2855.

Kremer, J. J. and R. M. Murphy (2003). "Kinetics of adsorption of beta-amyloid peptide A beta(1-40) to lipid bilayers." Journal of Biochemical and Biophysical Methods **57**(2): 159-169.

Kumar, M. N. V. R., R. A. A. Muzzarelli, et al. (2004). "Chitosan Chemistry and Pharmaceutical Perspectives." Chemical Reviews **104**(12): 6017-6084.

Lambert, M. P., A. K. Barlow, et al. (1998). "Diffusible, nonfibrillar ligands derived from Abeta 1-42 are potent central nervous system neurotoxins." Proceedings of the National Academy of Sciences of the United States of America **95**(11): 6448-6453.

Landers, J. J., Z. Y. Cao, et al. (2002). Prevention of influenza pneumonitis by sialic acid-conjugated dendritic polymers.

Lesne, S., M. T. Koh, et al. (2006). "A specific amyloid-[beta] protein assembly in the brain impairs memory." Nature **440**(7082): 352-357.

Li, B., M. Chohan, et al. (2007). "Disruption of microtubule network by Alzheimer abnormally hyperphosphorylated tau." Acta Neuropathologica **113**(5): 501-511.

Lin, H., R. Bhatia, et al. (2001). "Amyloid {beta} protein forms ion channels: implications for Alzheimer's disease pathophysiology." FASEB J. **15**(13): 2433-2444.

Lin, M.-S., L.-Y. Chen, et al. (2008). "Examining the levels of ganglioside and cholesterol in cell membrane on attenuation the cytotoxicity of beta-amyloid peptide." Colloids and Surfaces B: Biointerfaces **65**(2): 172-177.

Lindsay, J., D. Laurin, et al. (2002). "Risk Factors for Alzheimer's Disease: A Prospective Analysis from the Canadian Study of Health and Aging." Am. J. Epidemiol. **156**(5): 445-453.

Liu, Y. B., D. A. Peterson, et al. (1997). "Mechanism of cellular 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction." Journal of Neurochemistry **69**(2): 581-593.

Lleo, A. (2007). "Current therapeutic options for Alzheimer's disease." Current Genomics **8**(8): 550-558.

Lloyd, K. O. and K. Furukawa (1998). "Biosynthesis and functions of gangliosides: recent advances." Glycoconjugate Journal **15**(7): 627-636.

Luchsinger, J. A. and R. Mayeux (2004). "Dietary factors and Alzheimer's disease." The Lancet Neurology **3**(10): 579-587.

M.Hoozemans, J. J., S. M. Chafekar, et al. (2006). "Always Around, Never the Same: Pathways of Amyloid Beta Induced Neurodegeneration Throughout the Pathogenic Cascade of Alzheimer's Disease." Current Medicinal Chemistry **13**(22): 2599-2605.

Makimura, Y., S. Watanabe, et al. (2006). "Chemoenzymatic synthesis and application of a sialoglycopolymer with a chitosan backbone as a potent inhibitor of human influenza virus hemagglutination." Carbohydrate Research **341**(11): 1803-1808.

Mandal, P. and J. Pettegrew (2004). "Alzheimer's Disease: NMR Studies of Asialo (GM1) and Trisialo (GT1b) Ganglioside Interactions with Aβ(1-40) Peptide in a Membrane Mimic Environment." Neurochemical Research **29**(2): 447-453.

Mandavilli, A. (2006). "The amyloid code." Nat Med **12**(7): 747-751.

Masters, C. L., G. Simms, et al. (1985). "Amyloid plaque core protein in Alzheimer disease and Down syndrome." Proceedings of the National Academy of Sciences of the United States of America **82**(12): 4245-4249.

Matsuzaki, K. (2007). "Physicochemical interactions of amyloid [beta]-peptide with lipid bilayers." Biochimica et Biophysica Acta (BBA) - Biomembranes **1768**(8): 1935-1942.

Matsuzaki, K. and C. Horikiri (1999). "Interactions of Amyloid-beta Peptide (1-40) with Ganglioside containing Membranes." Biochemistry **38**(13): 4137-4142.

Mattson, G., E. Conklin, et al. (1993). "A Practical Appraoch to Cross-Linking." Molecular Biology Reports **17**(3): 167-183.

Mayeux, R. (2003). "Epidemiology of Neurodegeneration." Annual Review of Neuroscience **26**(1): 81-104.

Mayeux, R., A. M. Saunders, et al. (1998). "Utility of the Apolipoprotein E Genotype in the Diagnosis of Alzheimer's Disease." N Engl J Med **338**(8): 506-511.

McConnell, L. M., G. D. Sanders, et al. (1999). Evaluation of genetic tests: APOE genotyping for the diagnosis of Alzheimer disease, Mary Ann Liebert Inc Publ.

McLaurin, J. and A. Chakrabartty (1996). "Membrane Disruption by Alzheimer beta -Amyloid Peptides Mediated through Specific Binding to Either Phospholipids or Gangliosides. IMPLICATIONS FOR NEUROTOXICITY." J. Biol. Chem. **271**(43): 26482-26489.

McLaurin, J. and A. Chakrabartty (1997). "Characterization of the interactions of Alzheimer beta-amyloid peptides with phospholipid membranes." European Journal of Biochemistry **245**(2): 355-363.

McLaurin, J., T. Franklin, et al. (1998). "Structural Transitions Associated with the Interaction of Alzheimer beta -Amyloid Peptides with Gangliosides." J. Biol. Chem. **273**(8): 4506-4515.

Minati, L., T. Edginton, et al. (2009). "Reviews: Current Concepts in Alzheimer's Disease: A Multidisciplinary Review." American Journal of Alzheimer's Disease and Other Dementias **24**(2): 95-121.

Mishra, V., U. Gupta, et al. (2009). "Surface-Engineered Dendrimers: a Solution for Toxicity Issues." Journal of Biomaterials Science-Polymer Edition **20**(2): 141-166.

Mosmann, T. (1983). "Rapid Colorimetric Assay for Cellular Growth and Survival: Application to Proliferation and Cyto-Toxicity Assays." Journal of Immunological Methods **65**(1-2): 55-63.

Multhaup, G., T. Ruppert, et al. (1997). "Reactive oxygen species and Alzheimer's disease." Biochemical Pharmacology **54**(5): 533-539.

Muzzarelli, R. A. A. and C. Muzzarelli (2005). Chitosan chemistry: Relevance to the biomedical sciences. Polysaccharides 1: Structure, Characterization and Use. **186:** 151-209.

Nathalie, P. and O. Jean-Noel (2008). "Processing of Amyloid Precursor Protein and Amyloid Peptide Neurotoxicity." Current Alzheimer Research **5**(2): 92-99.

Nerelius, C., J. Johansson, et al. (2009). "Amyloid beta-peptide aggregation. What does it result in and how can it be prevented?" Frontiers in Bioscience **14**: 1716-U3856.

Nordberg, A. (2007). "Amyloid imaging in Alzheimer's disease." Current Opinion in Neurology **20**(4): 398-402.

Okennedy, R. (1979). "Some observations on Sialic Acid determination by the Warren Thiobarbituric Acid Method." Irish Journal of Medical Science **148**(3): 92-96.

Paravastu, A. K., I. Qahwash, et al. (2009). "Seeded growth of beta-amyloid fibrils from Alzheimer's brain-derived fibrils produces a distinct fibril structure." Proceedings of the National Academy of Sciences of the United States of America **106**(18): 7443-7448.

Parihar, M. S. and T. Hemnani (2004). "Alzheimer's disease pathogenesis and therapeutic interventions." Journal of Clinical Neuroscience **11**(5): 456-467.

Patel, D., J. Henry, et al. (2006). "Attenuation of [beta]-amyloid induced toxicity by sialic acid-conjugated dendrimeric polymers." Biochimica et Biophysica Acta (BBA) - General Subjects **1760**(12): 1802-1809.

Patel, D. A. (2007). Understanding the mechanism of preventing beta-amyloid induced neurotoxicity in Alzheimer's Disease. Department of Chemical and Biochemical Engineering, University of Maryland. **Ph.D.:** 4-24.

Patel, D. A., J. E. Henry, et al. (2007). "Attenuation of [beta]-amyloid-induced toxicity by sialic-acid-conjugated dendrimers: Role of sialic acid attachment." Brain Research **1161**: 95-105.

Pedersen, J. S. and D. E. Otzen (2008). "Amyloid - a state in many guises: Survival of the fittest fibril fold." Protein Science **17**(1): 2-10.

Petkova, A. T., Y. Ishii, et al. (2002). "A structural model for Alzheimer's beta- amyloid fibrils based on experimental constraints from solid state NMR." Proceedings of the National Academy of Sciences of the United States of America **99**(26): 16742-16747.

Pillai, C. K. S., W. Paul, et al. (2009). "Chitin and chitosan polymers: Chemistry, solubility and fiber formation." Progress in Polymer Science **34**(7): 641-678.

Pimplikar, S. W. (2009). "Reassessing the amyloid cascade hypothesis of Alzheimer's disease." The International Journal of Biochemistry & Cell Biology **41**(6): 1261-1268.

Plant, L. D., J. P. Boyle, et al. (2003). "The Production of Amyloid {beta} Peptide Is a Critical Requirement for the Viability of Central Neurons." J. Neurosci. **23**(13): 5531-5535.

Praticò, D. (2008). "Oxidative stress hypothesis in Alzheimer's disease: a reappraisal." Trends in Pharmacological Sciences **29**(12): 609-615.

Raafat, D., K. von Bargen, et al. (2008). "Insights into the mode of action of chitosan as an antibacterial compound." Applied and Environmental Microbiology **74**(12): 3764-3773.

Rauk, A. (2008). "Why is the amyloid beta peptide of Alzheimer's disease neurotoxic?" Dalton Transactions(10): 1273-1282.

Ravi Kumar, M. N. V. (2000). "A review of chitin and chitosan applications." Reactive and Functional Polymers **46**(1): 1-27.

RD Terry, R. K., KL Bick (1994). Diagnosis, in Alzheimer's Disease, Raven Pr.

Reuter, J. D., A. Myc, et al. (1999). "Inhibition of Viral Adhesion and Infection by Sialic-Acid-Conjugated Dendritic Polymers." Bioconjugate Chemistry **10**(2): 271-278.

Roland Jakob-Roetne, H. J. (2009). "Alzheimer's Disease: From Pathology to Therapeutic Approaches." Angewandte Chemie International Edition **48**(17): 3030-3059.

Rymer, D. L. and T. A. Good (2001). "The role of G protein activation in the toxicity of amyloidogenic A beta-(1-40), A beta-(25-35), and bovine calcitonin." Journal of Biological Chemistry **276**(4): 2523-2530.

Scarpini, E., P. Schelterns, et al. (2003). "Treatment of Alzheimer's disease; current status and new perspectives." The Lancet Neurology **2**(9): 539-547.

Scheltens, P., N. Fox, et al. (2002). "Structural magnetic resonance imaging in the practical assessment of dementia: beyond exclusion." The Lancet Neurology **1**(1): 13-21.

Schenk, D., R. Barbour, et al. (1999). "Immunization with amyloid-[beta] attenuates Alzheimer-disease-like pathology in the PDAPP mouse." Nature **400**(6740): 173-177.

Scheuner, D., C. Eckman, et al. (1996). "Secreted amyloid [beta]-protein similar to that in the senile plaques of Alzheimer's disease is increased in vivo by the presenilin 1 and 2 and APP mutations linked to familial Alzheimer's disease." Nat Med **2**(8): 864-870.

Schladitz, C., E. P. Vieira, et al. (1999). "Amyloid-[beta]-Sheet Formation at the Air-Water Interface." Biophysical Journal **77**(6): 3305-3310.

Schwarz, A. and A. H. Futerman (1996). "The localization of gangliosides in neurons of the central nervous system: the use of anti-ganglioside antibodies." Biochimica et Biophysica Acta (BBA) - Reviews on Biomembranes **1286**(3): 247-267.

Selkoe, D. J. (2002). "Alzheimer's Disease Is a Synaptic Failure." Science **298**(5594): 789-791.

Seow, D. and S. Gauthier (2007). "Pharmacotherapy of Alzheimer Disease." Canadian Journal of Psychiatry **52**(10): 620-629.

Serpell, L. C. (2000). "Alzheimer's amyloid fibrils: structure and assembly." Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease **1502**(1): 16-30.

Simons, K. and E. Ikonen (1997). "Functional rafts in cell membranes." Nature **387**(6633): 569-572.

Skovronsky, D. M., V. M. Y. Lee, et al. (2006). "Neurodegenerative Diseases: New Concepts of Pathogenesis and Their Therapeutic Implications." Annual Review of Pathology: Mechanisms of Disease **1**(1): 151.

Smith, M. A., P. L. R. Harris, et al. (1997). "Iron accumulation in Alzheimer disease is a source of redox-generated freeâ€‰radicals." Proceedings of the National Academy of Sciences of the United States of America **94**(18): 9866-9868.

Snowdon, D. A., S. J. Kemper, et al. (1996). "Linguistic ability in early life and cognitive function and Alzheimer's disease in late life. Findings from the Nun Study." JAMA **275**(7): 528-532.

Sonnino, S., L. Mauri, et al. (2007). "Gangliosides as components of lipid membrane domains." Glycobiology **17**(1): 1R-13.

Soto, C. (1999). "Plaque busters: strategies to inhibit amyloid formation in Alzheimer's disease." Molecular Medicine Today **5**(8): 343-350.

Soto, C., E. M. Sigurdsson, et al. (1998). "beta-sheet breaker peptides inhibit fibrillogenesis in a rat brain model of amyloidosis: Implications for Alzheimer's therapy." Nature Medicine **4**(7): 822-826.

Stokin, G. B., C. Lillo, et al. (2005). "Axonopathy and Transport Deficits Early in the Pathogenesis of Alzheimer's Disease." Science **307**(5713): 1282-1288.

Tansley, G. H., B. L. Burgess, et al. (2007). "The cholesterol transporter ABCG1 modulates the subcellular distribution and proteolytic processing of {beta}-amyloid precursor protein." J. Lipid Res. **48**(5): 1022-1034.

Teng, F. Y. H. and B. L. Tang (2005). "Widespread [gamma]-secretase activity in the cell, but do we need it at the mitochondria?" Biochemical and Biophysical Research Communications **328**(1): 1-5.

Terzi, E., G. Holzemann, et al. (1994). "Alzheimer Beta-Amyloid Peptide 25-35: Electrostatic Interactions with Phospholipid Membranes." Biochemistry **33**(23): 7434-7441.

Terzi, E., G. Holzemann, et al. (1997). "Interaction of Alzheimer beta-amyloid peptide(1-40) with lipid membranes." Biochemistry **36**(48): 14845-14852.

Terzi, E., G. Hölzemann, et al. (1995). "Self-association of [beta]-Amyloid Peptide (1-40) in Solution and Binding to Lipid Membranes." Journal of Molecular Biology **252**(5): 633-642.

The Ronald and Nancy Reagan Research Institute of the Alzheimer's Association and National Institute on Aging Working Group (1998). "Consensus Report of the Working Group on: "Molecular and Biochemical Markers of Alzheimer's Disease"." Neurobiology of Aging **19**(2): 109-116.

Tjernberg, L. O., D. J. E. Callaway, et al. (1999). "A Molecular Model of Alzheimer Amyloid beta -Peptide Fibril Formation." J. Biol. Chem. **274**(18): 12619-12625.

Tjernberg, L. O., J. Naslund, et al. (1996). "Arrest of beta-amyloid fibril formation by a pentapeptide ligand." Journal of Biological Chemistry **271**(15): 8545-8548.

Umemura, M., M. Itoh, et al. (2008). "Design of a Sialylglycopolymer with a Chitosan Backbone Having Efficient Inhibitory Activity against Influenza Virus Infection." Journal of Medicinal Chemistry **51**(15): 4496-4503.

Verdier, Y. and B. Penke (2004). "Binding Sites of Amyloid beta-Peptide in Cell Plasma Membrane and Implications for Alzheimer's Disease." Current Protein & Peptide Science **5**(1): 19-31.

Villemagne, V. L., R. Cappai, et al. (2007). The Aßcentric Pathway of Alzheimer's Disease. Abeta Peptide and Alzheimer's Disease:Celebrating a Century of Research. C. J. Barrow and D. H. Small.

Wakabayashi, M., T. Okada, et al. (2005). "GM1 ganglioside-mediated accumulation of amyloid [beta]-protein on cell membranes." Biochemical and Biophysical Research Communications **328**(4): 1019-1023.

Walsh, D. M., D. M. Hartley, et al. (1999). "Amyloid beta -Protein Fibrillogenesis. Structure and Biological Activity of Protofibrillar Intermediates." J. Biol. Chem. **274**(36): 25945-25952.

Walsh, D. M., A. Lomakin, et al. (1997). "Amyloid beta -Protein Fibrillogenesis. Detection of a Protofibrillar Intermediate." J. Biol. Chem. **272**(35): 22364-22372.

Walsh, D. M. and D. J. Selkoe (2007). "A-beta Oligomers - a decade of discovery." Journal of Neurochemistry **101**(5): 1172-1184.

Wang, J., D. W. Dickson, et al. (1999). "The Levels of Soluble versus Insoluble Brain A[beta] Distinguish Alzheimer's Disease from Normal and Pathologic Aging." Experimental Neurology **158**(2): 328-337.

Wang, S. S. S., D. L. Rymer, et al. (2001). "Reduction in cholesterol and sialic acid content protects cells from the toxic effects of beta-amyloid peptides." Journal of Biological Chemistry **276**(45): 42027-42034.

Warren, L. (1959). "The Thiobarbituric Acid Assay of Sialic Acids." J. Biol. Chem. **234**(8): 1971-1975.

Waschuk, S. A., E. A. Elton, et al. (2001). "Cellular Membrane Composition Defines Abeta - Lipid Interactions." J. Biol. Chem. **276**(36): 33561-33568.

Williamson, M. P., Y. Suzuki, et al. (2006). "Binding of amyloid beta-peptide to ganglioside micelles is dependent on histidine-13." Biochem J **397**(3): 483-490.

Wilquet, V. and B. D. Strooper (2004). "Amyloid-beta precursor protein processing in neurodegeneration." Current Opinion in Neurobiology **14**(5): 582-588.

Yanagisawa, K. (2007). "Role of gangliosides in Alzheimer's disease." Biochimica et Biophysica Acta (BBA) - Biomembranes **1768**(8): 1943-1951.

Yanagisawa, K. and Y. Ihara (1998). GM1 ganglioside-bound amyloid beta-protein in Alzheimer's disease brain, Elsevier Science Inc.

Yanagisawa, K., A. Odaka, et al. (1995). "GM1 ganglioside-bound amyloid beta-protein (Abeta): A possible form of preamyloid in Alzheimer's disease." Nat Med **1**(10): 1062-1066.

Yankner, B. A. and T. Lu (2009). "Amyloid {beta}-Protein Toxicity and the Pathogenesis of Alzheimer Disease." J. Biol. Chem. **284**(8): 4755-4759.

Zheng, H., M. Jiang, et al. (1995). "[beta]-amyloid precursor protein-deficient mice show reactive gliosis and decreased locomotor activity." Cell **81**(4): 525-531.

Zubenko, G., B. Cohen, et al. (1987). "Platelet membrane fluidity in Alzheimer's disease and major depression." Am J Psychiatry **144**(7): 860-868.

VITA

Dhruva Dilip Dhavale was born in Pune, India, in December 1984. After completing his high school and secondary college, he graduated from University of Pune, India, with a Bachelor of Engineering degree in chemical engineering discipline in August 2006. After working at National Chemical Laboratory, Pune, as a project assistant for one year, Dhruva was admitted into the Graduate program of the Department of Chemical Engineering at Louisiana State University at Baton Rouge. There he worked on the development of sialic acid labeled chitosan for the attenuation of amyloid-beta toxicity under the guidance of Dr. James Henry, and will receive his Master of Science in Chemical Engineering degree at the Fall 2009 commencement. He plans to continue with Dr. James Henry at LSU for his doctorate.